Development of New Tritium Labelling Methods for Peptides & Investigation of Guest-Host Mediated Electrocyclization and Sigma-Tropic Rearrangement Reactions

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Development of New Tritium Labelling Methods for Peptides

&

Investigation of Guest-Host Mediated Electrocyclization and Sigma-Tropic Rearrangement Reactions

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Risø-PhD-59(EN)

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Dedicated to Past and Future
-Radiochemistry Students and Researchers.

Preface

This thesis is a result of three years of graduate work performed at the Hevesy laboratory, Risø National Laboratory, The Technical University of Denmark, under the supervision of Dr. Lars Martiny, Head of the Radiation Research Division.

The main parts of the work presented here is Part I; which have involved the installation of a Tritium Chemistry Facility for the synthesis of radiolabelled compounds with tritium, and Part II; the development of new tritium labelling methods for peptides.

The intention of Part I is to supply background information of radioactivity in general, tritium uses in research and development and in the result section of Part I the installed Hevesy Tritium Facility (HTF) is introduced.

One of the overall objectives for this work was investigation of new tritium labelling techniques for peptides. The background and results from this research is presented in Part II and the Appendix I.

Finally, a substantial part (over a period of 8 months) of the research presented in this thesis was performed in the Bergman group, University of California, Berkeley. As a visiting scholar, I was involved in investigation of Host-Guest (supramolecular) chemistry in joint project collaboration between Prof. Robert G. Bergman and Prof. Kenneth Raymond. Part III of this thesis is the research report from my external research visit and it can be read as an independent Part.

Acknowledgements

I am very grateful to many people who have helped and inspired me throughout my graduate works. I would like especially like to acknowledge my tutor of radiochemistry work and supervisor **Dr. Lars Martiny,** who have supplied me with excellent knowledge about tritium and radiochemistry in general.

Furthermore I would like to express my gratitude to the following persons;

Prof. Kai Mikael Jensen for his great inspirations and inputs about the installation of the Hevesy tritium facility and helping with acquiring the necessary licenses and instruments for working with multicurie quantities of tritium.

The Novo Nordisk Isotope Group; **Dr. Uffe Larsen**, **Dr. Jesper B. Kristensen**, **Heidi Hansen**, for sharing their experiences and tutoring me for working with tritium and installing a tritium manifold system as well as the supply of the iodinated-GLP-1 substrate peptide.

The laboratory technicians **Henrik Prip** and **Lasse Hauerberg** for their help with installing the tritium manifold system.

Dr. Palle Rasmussen for his inputs about the synthesis of polypeptides and instructing me on the instrumental necessities for these syntheses.

My greatest gratitude goes to my fellow workers at The Hevesy Laboratory, Risø and UCB for making these three years an experience of a lifetime! Especially I'd like to thank my current office mates; **Dr. Kristian Jensen**, **Andreas Jensen** and **Alex Givskov** for support, proof reading and always interesting discussions in the office/laboratory.

I would also like to acknowledge my collaborators at the University of Copenhagen. Especially **Dr. Petrine Wellendorph** and **Dr. Signe Høg** for the investigation and synthesis of precursors for GHB radioligands.

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Appendix II. *Proceeding: Radioiododestannyllation of Novel GHB Receptor Ligands*

Part III. ***Investigation of Guest-Host Mediated Electro-cyclization and Sigmatropic Rearrangement Reactions***

Manuscripts

Selective Deuteriodeiodination of Iodinated Tyrosine in Peptides using Deuterated Triethylsilane and Homogenous Pd(0)
Submitted to Journal for Radiolabelled Compounds and Radiopharmaceuticals, 2010.
Martin Pedersen and Lars Martiny.

Radioiododestannyllation of Novel GHB Neuroreceptor Ligands.
Martin Pedersen, Signe Høg, Petrine Wellendorph and Lars Martiny.
Journal of Labelled Compounds and Radiopharmaceuticals, **2010,** 53, (5-6), 318-320.
Proceedings for the 10th International Isotope Society Symposium, Chicago, 2009.

Other Manuscripts Currently in Press

Novel radioiodinated GHB analogues for radiolabelling and photolinking of high-affinity GHB binding sites
Petrine Wellendorph, Signe Høg, Paola Sabbatini, Martin H. F. Pedersen, Lars Martiny, Gitte Moos Knudsen, Bente Frølund, Rasmus P. Clausen, and Hans Bräuner-Osborne.
Journal of Pharmacology and Experimental Therapeutic, **2010**, Article ASAP.

Design, Synthesis, and in Vitro Pharmacology of New Radiolabeled γ-Hydroxybutyric Acid Analogues Including Photolabile Analogues with Irreversible Binding to the High-Affinity γ-Hydroxybutyric Acid Binding Sites
Paola Sabbatini, Petrine Wellendorph, Signe Høg, Martin H. F. Pedersen, Hans Bra□uner-Osborne, Lars Martiny, Bente Frølund, Rasmus P. Clausen.
Journal of Medicinal Chemistry, **2010**, Article ASAP.

Posters

New Methods for the Efficient Tritium Labelling of Peptides and Proteins
Danish Chemical Society, Odense, Denmark, 2007.
Martin Pedersen and Lars Martiny.

I-125 Labelling and the Application of Novel GHB Receptor Ligands
International Isotope Society Symposium, Chicago, USA, 2009.
Martin Pedersen, Signe Høg, Petrine Wellendorph and Lars Martiny.

Selected Oral Presentations

Tritium Labelling of Peptides and Proteins
at the Danish Radiochemical Meeting, Roskilde, Denmark, 2007.
Martin Pedersen.

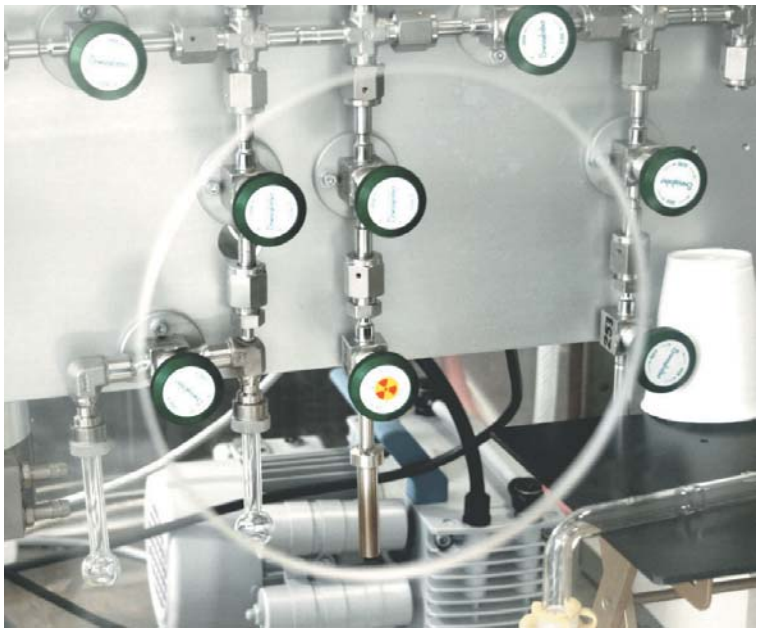
Tritium; Characteristics, Sources and Problems
T_2 Chemistry; Methodologies and Waste Handling
for the Danish Decommissioning, Roskilde, Denmark, 2008.
Martin Pedersen.

I-125 Labelling of Neuroreceptor Ligands
at the 10th International Isotope Society Symposium, Chicago, USA, 2009.
Martin Pedersen.

Multicurie Tritium Chemistry on a Tritium Manifold System
at the Danish Radiochemical Meeting, Copenhagen, Denmark 2010.
Martin Pedersen.

Part I.

Introduction to Radioactivity and Tritium as a *workhorse* in Pharmaceutical R&D



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Front page: Illustration of the uranium storage bed for tritium mounted onto the Tritium Manifold System. This bed contains 250 Ci (9.25 TBq) of tritium stored as uranium tritide.

Objectives and Aims

The objectives of Part I of this thesis in to provide an introduction to radioactivity in general, and to tritium as it is indeed a *workhorse* in (pharmaceutical) research and development.

The radiohydrogen isotope, tritium, has generally found many useful applications. In life sciences and in the pharmaceutical industry tritium functions as the *workhorse* as a tracer for new chemical entities and this isotope is essential as a tool for early ADME studies.

A significant part of the graduate studies presented here, has involved the acquisition of laboratory instrumentation for performing of gaseous tritium chemistry. The process of setting up the Hevesy Laboratory's tritium labelling facility as well as necessary analytical instruments will be described in detail. This also mean, that this part will function as a materials and methods section for the second part of this thesis; "Development of New Tritium Labelling Methods for Peptides".

The aim of this part is also to function as an introduction to the tritium isotope; here the general/current tritium (T_2) chemistry methodologies, for labelling of small chemical compounds, are briefly overviewed. One example of isotopic scrambling in tritiation of an unsaturated compounds and the general mechanism of this phenomena will be proposed as a part of the results section.

Background

Radioactivity

The term “Radio-activity” was introduced by, the pioneers of radioactive elements, Henri Becquerel, Marie Curie and Pierre Curie. In 1903 these three individuals were recognised for their work by sharing the Nobel Prize in physics[1, 2].

In general, non-stable atomic isotopes will disintegrate into more stable elements. This nuclidic disintegration can occur in different pathways depending on the isotope/nuclidic composition and energy levels. A result of the nuclidic disintegration is, in all cases, emission of either particles (neutrons, protons or electrons) or electromagnetic radiation. This phenomenon is in general known as Radioactivity.

The terminology of related to the different radioactive decays is listed in **table 1**.

Terminology	Abbreviation	Process
Beta Decay	β^- and β^+	Emission of a beta particle (negatron or positron) from the nucleus.
Electron Capture	**ε / EC**	Nuclidic capture of an inner shell electron.
Gamma Ray	**γ**	Electromagnetic emission from the nucleus.
X-ray or Röntgen		Electromagnetic emission from the electron shell surrounding the nucleus.
Internal Conversion	**IC**	Electron emission from interaction of the nucleus and an inner shell electron.
Auger Electron		Conversion electron emitted from the electron shells.
Alfa Particle	**α**	Emission of an alpha particle ($^4_2$He) from the nucleus of the parent isotope.
Isomeric Transition	**IT**	Nuclidic evaporation of energy (γ) from a meta-stable isotope, to leave the nucleus in a lower energy state.
Spontaneous Fission	**SF**	Spontaneous spallatation of a nuclide for formation of two or more daughter nuclides

Table 1. General terminology of decay pathway in radioactive disintegrations.

All elements of the periodic table have radioactive isotopes. An isotope of the same element in the periodic table, have the same number of protons (p^+) and different number of neutrons (n) hence the word isotope. Actually the word isotope originates from the greek words *iso topos* which means “same place”. In general, neutron

deficient nuclides tend to disintegrate either by EC ($p^+ + e^- \rightarrow n$) or positron emission ($p \rightarrow n + \beta^+$) and neutron rich nuclides by negatron emission ($n \rightarrow p^+ + \beta^-$).

Isotopes of the same element are generally considered to have the same chemical properties and behave similar chemically. (This is though not completely true due to isotope effects and different vibrational energy states of the chemical bonds). This is why radioactive isotopes can be used as tracers for the stable counterparts.

Radioactivity is measured in disintegrations (the number of decaying nuclides) in a given timeframe. The SI unit for radioactive amounts is Becquerel (Bq) which means the amount of disintegrations per second. Another widely used (especially with tritium) unit for radioactive amounts is Curie (Ci). One Curie is standardized from the total disintegrations from 1 gram of radium-226 and equals $37*10^9$ Bq.

The Beta Decay

The energy profile for an emitted beta particle (β^- or β^+) is characteristic for each radionuclide and this energy profile can be illustrated by liquid scintillation counting (LSC). The kinetic energy of the emitted beta particle has a continuous spectrum, for tritium the mean energy is 5.7 keV and a maximum energy of 18.6 keV. Compared to other radioisotopes this is a very low energy (in the range of Auger electron energies) and this make tritium difficult to detect by other methods than scintillation. The emitted particle from tritium has a maximum range of 6 mm in air. Detection of tritium is therefore normally performed by liquid scintillation counting. In figure 1 a few β spectra are illustrated made by LSC.

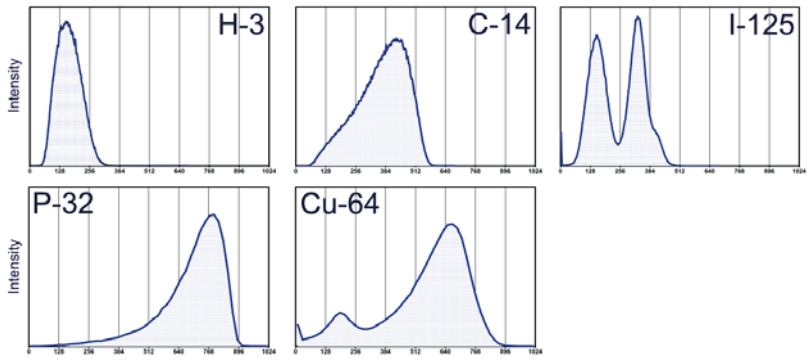


Figure 1. Illustration of beta-decay energy profiles of five important radioisotopes used in research and development. These spectrums are acquired on a Wallac Rack-beta 1412 liquid scintillation counter. The energy profiles are plottet counts (intensity) by channel (with a 1024 multi channel analyser). All the isotopes here emit negatron particles, Cu-64 i the only nuclide emitting both negatrons and positrons.

The Radiotracer Principle and Application of Tracers

"*The radiotracer method is clearly one of the most powerful tools in scientific research*", Wang and Willis, 1963.

The above citation of Wang and Willis[3] could arguably be somewhat of an exaggeration, but the use of radioactive tracers is without doubt a very powerful and indispensable tool in science. Radioactive Indicators or Radiotracers used for the investigation of biological processes was first described by George de Hevesy in 1923. He described the application of radioactive lead isotopes and the distribution in various parts of plants simply by dipping them into a solution containing a radioactive isotope[4]. Later on (in 1943), he was the recipient of the Nobel Prize in chemistry for his work on the use of isotopes as tracers in the study of chemical processes[5].

Since radiolabelled compounds have (almost) the same physiochemical properties as the non-radioactive compounds of with the same elements, the radiolabelled compound can be used as tracers for the molecules in biological systems. Thereby, radiolabelled tracers provide an outstanding tool for the investigation of biological systems.

The radiotracer principle is widely used in administration, distribution, metabolism and excretion (ADME) studies for characterisation of new chemical entities[6, 7]. A part of the regulatory process and development of a new pharmaceutical is the requirement of studies of the distribution of the drug in experimental animals. Depending of the radioisotope used for these studies different detection instruments can be used. When tritiated compounds are used in *in-vivo* distribution studies - autoradiography is the method used. Whole Body Autoradiography (WBA) is a method for tracing a molecule in different parts and organs of an animal body. The WBA method involves the detection of radioactivity from thin biological slices from a frozen animal on a photographic sensitive plate.[8]

Another applicable method for the use of radiolabelled molecules is in receptor affinity studies. In large drug development programs, radiolabelled receptor ligands can be used for screening of large chemical libraries in High Throughput Screening (HTS). Displacement of known radiolabelled ligands toward a receptor target is one of the methods for evaluation of new potential ligands (antagonists or agonists).

During the last 3 years of studies I was also involved in the radiolabelling of a few neuroreceptor ligands with the iodine-125 isotope. One of many very useful radiolabelling methods is called the radioiododestannyllation reaction; this will later in this thesis be briefly reviewed and one example of this method has been presented (Appendix II). These radioligands synthesised as a part of this graduate work, are currently under investigation in receptor affinity and WBA/autoradiography studies[9, 10].

The Hydrogen Isotopes

Hydrogen-1 is the far most abundant element in our solar system and it is also the simplest atom having only one proton in its nucleus. The three most important isotopes of hydrogen are protium (P or ^{1}H), deuterium (D or ^{2}H) and tritium (T or ^{3}H). Tritium is a non-stable isotope with one proton and two neutrons in its nucleus and disintegrates by emission of a beta particle ($^{3}H \rightarrow {}^{3}He + \beta^{-}$). In **table 2**, the mentioned isotopes are listed.

The Hydrogen (H) Isotopes		
Isotope	**Natural Abundance**	**Halflife and Decay Properties**
^{1}H / P	99.9885 %	Stable
^{2}H / D	0.0115 %	Stable
^{3}H / T	Trace	12.33 years, pure β- emission (e_{max} = 18.7 keV and e_{mean} = 5.7 keV)

Table 2. Overview of the hydrogen isotopes.

Whilst protium and deuterium are found on the earth, tritium is only found in trace amounts. The natural occurring tritium arises from nuclear processes in the earth crust and by nuclear reactions happening when charged particles impact the earth's atmosphere. The main part of tritium on the earth originates from the nuclear detonations happening in the atom-bomb-race-age, where several atmospheric nuclear detonations caused the injection of tritium into the earth's environment. The minimum estimated world inventory of tritium has been calculated as $15 \bullet 10^{9}$ GBq (405 MCi ~ 4700 kg T) while the natural equilibrium is ~ 70 MCi. The vapour, HTO, is formed readily and is the most commonly encountered form of tritium in the environment.[11]

Formation of a tritium nucleus can occur in many nuclear spallatation processes, in SF or particle collisions. Also the production of high amount of tritium is yielded by neutron bombardment of lithium-6 ($^{6}Li + n \rightarrow {}^{4}He + {}^{3}H$). This production method may be used as fuel breeding in thermonuclear experimental fusion reactors (TER)[12]. An example of ternary fission is illustrated in **figure 2**.

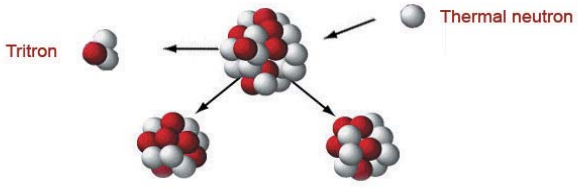


Figure 2. Illustration of a ternary fission process when a nuclide is bombarded with thermal neutrons. In this illustration two heavy daughter and tritron nuclides are produced.

Working with high amounts of gaseous tritium places a demand for special laboratory equipment as well as special licenses from the regulatory authorities[13, 14]. In the installation of a tritium facility, surveillance of contamination levels on surfaces and radioactivity in the air is a minimum requirement. A significant part of these graduate studies has involved the installation of tritium monitoring instruments for safe handling of tritium and also acquiring the permissions for the stockholding of up to 10 TBq tritium stored on uranium (see the results section).

The general objective of tritium (and radioactivity in general) safe handling is to prevent acute injurious radioactivity exposures, to ensure that regulatory limits are not exceeded and to keep exposures as low as reasonable achievable (ALARA). Tritium can be present in the workplace in airbourne forms as gas (T_2), vapour (HTO or T-solvents), dust or particles. The uptake of tritium in the human body can occur by inhalation, skin uptake or ingestion. For safety reasons it is therefore important that the tritium manifold is placed in a well ventilated fumehood.[15]

After uptake of a tritium containing compound the *in-vivo* distribution depends on the nature of the molecular bound tritium. Dosimetry-wise there is a clear separation between organically bound (OBT), gaseous (T_2) and aqueous tritium (HTO). The limit of the accumulated radioactivity dose is, in Denmark, 20 mSv per year for workers. This dose has been calculated and estimated to be equivalent to ingestion/inhalation of 1100 MBq (~30 mCi) of HTO or 490 MBq (~13 mCi) of OBT. The fate of a OBT *in-vivo* is of course dependent of the molecule labelled and the radioactive dose originating from these have to be evaluated from molecule to molecule. In **figure 3**, a model of the kinetic distribution for hydrogen in an average human body is illustrated. As it appear from the figure hydrogen can be modelled to be distributed into three hydrogen compartments, a central (H_2O) compartment and two organic bound hydrogen compartments. [11]

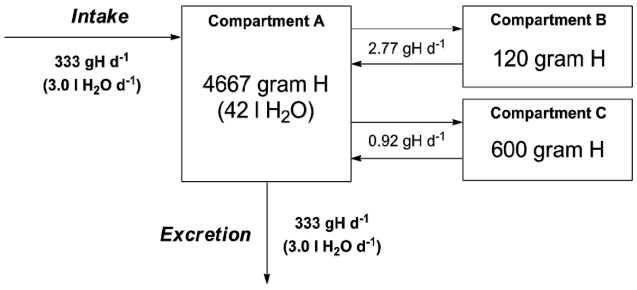


Figure 3. Illustration of a hydrogen (noted as H in the figure) distribution model of an average human body. Hydrogen is distributed into 3 compartments and has different pool sizes and kinetic exchange constants. Compartment A is the central water pool, compartment B and C the organic bound hydrogen.

If the presented three compartment model is applied in tritium kinetic calculations of HTO introduced into the human body – the amount of tritium can be calculated using Matlab software. For illustratory purposes the kinetic profile of ingested HTO is plotted against time (figure 4). As it appear the elimination of HTO is fast in the first phase having a biological half life of approximately 12 days. After tritium distribution into the peripheral compartments and bound to organic molecules (oligosaccharides, lipids, proteins and DNA/RNA) the elimination happens much slower.

When applying this model for HTO excretion, the first four biological half lifes of tritium can be calculated to 11.5 days, 31.3 days, 72 days and 127.2 days, where an amount of 1.3% (of the total ingestion) remains in the body after exposure.

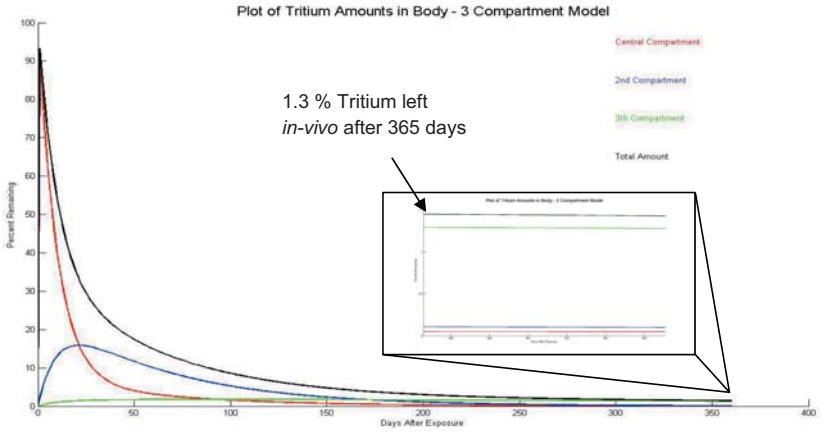


Figure 4. Plot of simulated of tritium distribution of consumed tritium water (HTO) in an average human generated in Matlab. The total tritium amount is plotted as the black line, central compartment (HTO) as the red line, and the two peripheral compartments as the blue and green lines.

General T_2 Tritium Chemistry

Tritium is a radioactive isotope of hydrogen and hence can provide valuable information if used in labelling of molecular tracers. Nearly all organic molecules and pharmaceuticals contain hydrogen. Tritium is therefore an ideal isotope for tracing organic molecules.

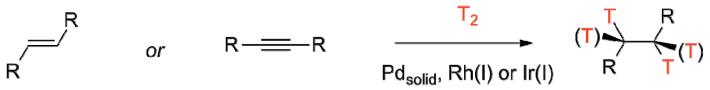
Practically all tritium labelled compounds are synthesised by the formation of a C-T bond because this is a relatively stable bond and C-T bonds are easy to create from gaseous tritium (T_2). It is necessary to form stable tritium bonds to ensure non labile tritium in the formed molecule. At the end of every tritiation reaction the labile tritium (*e.g.* formed N-T and O-T bond) is removed by repeated lyophillations of the product with protic solvents (e.g. a mixture of H_2O and MeOH)

General hydrog chemistry is well developed and tritium labelled compounds are normally synthesised by either tritide reductions (T^- + electrophile) or tritonations (T^+ + nucleophile). Many reaction types have been developed for tritium and have also been the topic for historical perspectives[16, 17], textbook[18] and review publications[19, 20]. Due to the many descriptions for incorporation of T into organic molecules only a brief overview of methodologies will be given here.

The first step in a (low pressure) gaseous tritiation reaction makes use of ditritium activation chemistry. This is normally performed by the application of platinum group metals (iridium, rhodium or palladium) which are excellent transition metals for dihydrogen activation chemistry. The general tritiation methodologies (and reaction types) is briefly illustrated in the following examples.

Aliphatic sp2-sp2 and sp-sp Bond Tritiation

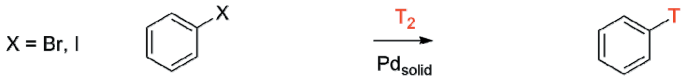
General hydrogenation chemistry of unsaturated multiple bonds is a frequently used for tritium labelling of small molecules. The overall reaction is illustrated in **scheme 14**. Varying tritium incorporations are often observed and this method can be used for synthesis of high specific activity compounds. An example of isotopic scrambling and the proposed mechanism for multiple tritium incorporations into an unsaturated compound is shown later in the result section. In a normal tritiation of a single double bond, the incorporation of one tritium is sometimes observed, leaving a specific activity of the labelled compound ~ 30 Ci/mmol (half of the expected).



Scheme 1. Illustration of a general tritiation (hydrogenation) with use of heterogeneous and homogeneous catalysts.

Tritiodehalogenation

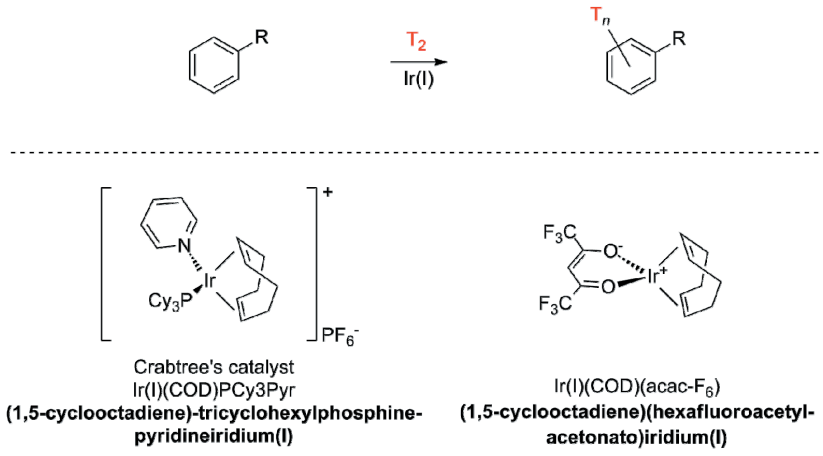
The tritiodehalogenation reaction (tritiolysis of a halogen-carbon bond) is another commonly used method for tritium labelling of aromatic compounds. In **scheme 2**, this general reaction is illustrated.



Scheme 2. Illustration of a general tritiodehalogenation (tritiolysis) reaction using heterogeneous palladium and tritium gas.

H/T exchange (Ir) Crabtree

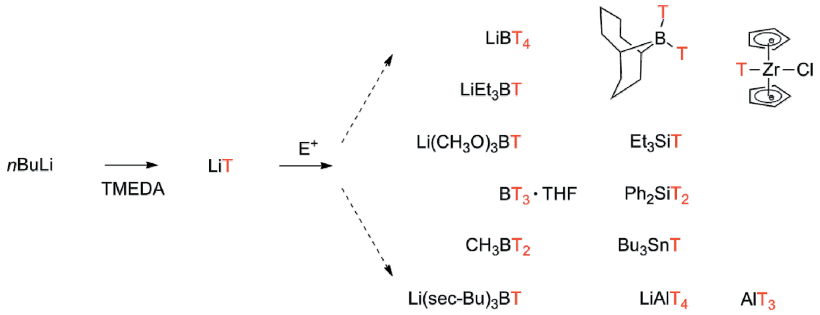
The discovery of C-H bond activation chemistry with organometallic complexes of iridium[21, 22] and utilizing these similar complexes with tritium and deuterium gas has revolutionised the area of isotopically exchange (H/T) reactions[16, 23]. In **scheme 3**, the general reaction of substituted aromatic compounds is illustrated. The incorporation of tritium is generally dependent on the aromatic substitution of the substrate. C-H activation is normally performed close/*ortho* to directing groups on aryls *e.g.* O or N directing groups[16].



Scheme 3. Illustration of the general H/T exchange reaction using organometallic iridium(I) complexes. Two commonly used iridium catalyst are shown in the figure.

nBuLi / TMEDA (Tritides)

With the use of a very strong base, such as n-butyllithium (*n*BuLi) in combination with *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TMEDA), it is also possible to perform acid-base chemistry on T_2. In this reaction tritiated butane and lithium tritide (LiT) are formed and the latter can be utilized in synthesis of complex tritides. In **scheme 4**, a selection of important tritium reactions are listed for the synthesis of exotic tritides for reductive tritiation.[19]



Scheme 4. Illustration of the synthesis of lithium tritide followed by reaction with a suitable electrophile for the synthesis of reducing reagents.

Results

In this section the results from the installation of a tritium manifold system and the setup of the Hevesy Laboratory's Tritium Facility is described in detail. A subsequent overview of tritium labelling of unsaturated phospholipids and the mechanism for isotopic scrambling is also given here.

Description of the Tritium Manifold System

The minimum demand for safe manipulation of high amounts of gaseous tritium is a manifold system. Design and acquiring of such a system was the first objective that had to be overcome for the work of this thesis. The results from installation of the Hevesy Laboratory Tritium Manifold are here illustrated, and this section can be read as a part of the materials and methods of the hydrogenation chemistry performed.

The deuterium and tritium reactions were all performed on a custom designed tritium manifold system manufactured by RC Tritec, Teufen, Switzerland. The tritium manifold is constructed of several parts for ensuring a safe handling of carrier-free gaseous tritium (T_2) in multi curie (Ci) amounts of radioactivity. It is primarily constructed with stainless steel tubing and Swagelok® high quality valves (figure 5). A picture of the Hevesy Laboratory's[24] tritium manifold is illustrated on figure 4, a picture of the backside on figure 6 and the schematic design with indication of calibrated tube volumes in figure 7.

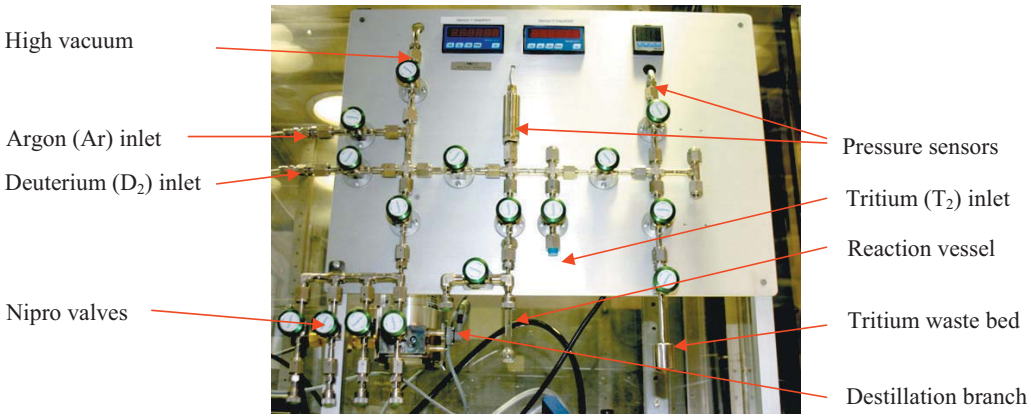


Figure 4. The picture shows the tritium manifold system with annotations of the different components without the mounted tritium source/inlet uranium bed.

The manifold is equipped with four pressure sensors; two Baumer® (0-4000 mbar) and two high vacuum Pirani (10^{-4} – 1000 mbar) sensors. The first Baumer® pressure sensors is for measurement of gaseous pressure in the central tube compartment and the second is for monitoring the pressure in the reaction vessel. A Pfeiffer (TMH

071) turbo molecular drag pump with a membrane forepump is placed on the back of the manifold for creation of high vacuum. The first Pirani pressure sensor is located in front of the turbo pump and the second on the manifold system. On two LCD displays on the top of the aluminium panel the pressure from the two Baumer® pressure sensors is displayed and on a separate Pfeiffer® total pressure controller, (outside of the fumehood) the high vacuum is displayed.

Tritium is stored on a uranium bed and is heated up to 500°C for release of T_2 gas. An additional uranium waste bed is used for absorption of radioactive hydrogen gas after reaction.

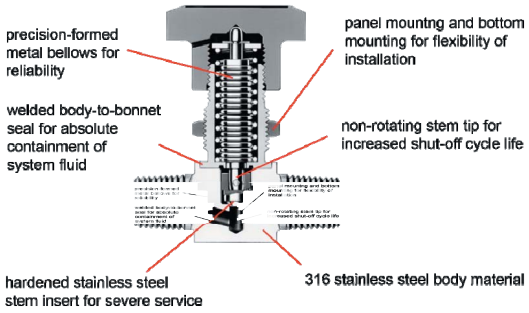


Figure 5. (*right*) Schematic drawing of a Swagelok® Nupro-SS-4-HTW bellow valve.

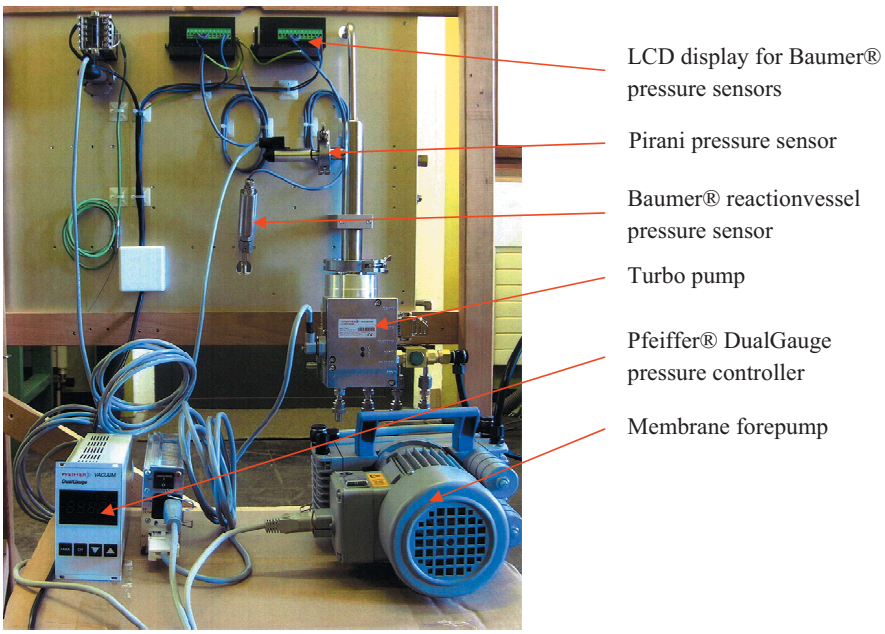


Figure 6. The back of the manifold is shown to illustrate wires, vacuum pumps and pressure sensors.

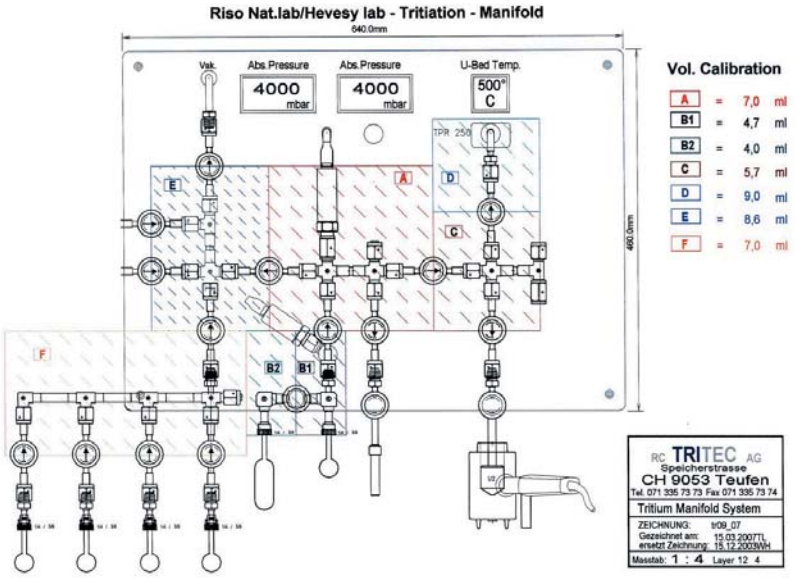


Figure 7. Schematic drawing of the Hevesy Laboratory's[24] tritium manifold system with information about the calibrated tube volumes.

The Hevesy Laboratory Tritium Facility

In installations of a tritium manifold in a laboratory several considerations regarding safety and practical issues must be undertaken. The placement in a well ventilated fumehood or in a glovebox is a requirement for minimising the possibility of contamination incidents. Furthermore, for the analysis of products formed in tritiation reactions analytical instruments have to be set up. Ideally HPLC, mass spectrometry and LSC instruments are placed in dedicated analysis laboratories. In figure 8, a picture of the installed manifold system in the fumehood is illustrated, indicating the individual parts relevant to the manifold.

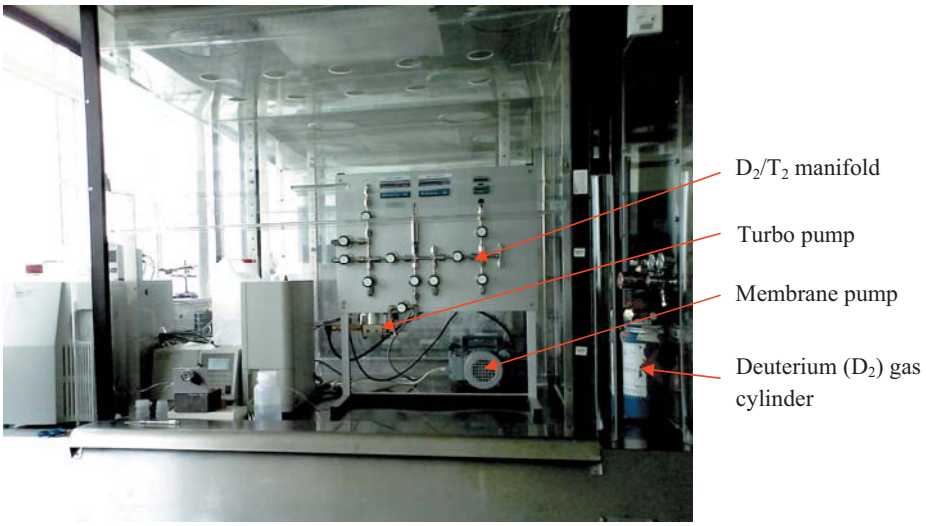


Figure 8. The laboratory setup of the tritium manifold system and deuterium gas cylinder for deuterium chemistry experiments is shown in the picture.

At the Hevesy laboratory, we chose to install a HPLC instrument inside the fumehood. This instrument is necessary for purification (preparative HPLC) and analysis of tritiated products. Here a Waters Breeze HPLC system was set up, with computer, binary pump and eluents outside of the fumehood. The injection port, UV detector and columns were all set up inside the fumehood. This installation (before applying tritium on the manifold) is illustrated on figure 9 on the next page.

For radioactivity issues this setup was appropriate, of practical reasons the parts placed inside the fumehood can be considered tritium contaminated. All utensils must thus be wipe tested for a contamination check, before leaving this area. Also this setup minimises the risk of contamination of other areas of the laboratory when performing the reaction, purification and analysis in the same fumehood in a small confined area.

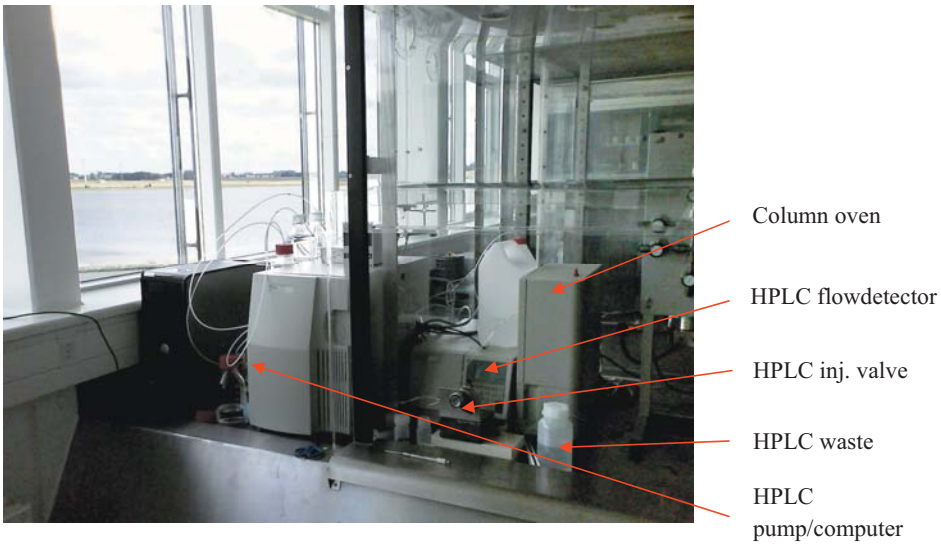


Figure 9. The picture shows the analytical and preparative HPLC system used for the deuterium and tritium experiments.

Installation of radioanalytical tools

When the fumehood and installation of the manifold were prepared for radioactivity, several changes of the fumehood were made, as well as, acquiring necessary radiodetectors for safety and analysis. In figure 10, the final setup of the tritium facility is illustrated. For safety and detection of gaseous tritium, a Canberra tritium *in-air* monitor was placed outside the fumehood equipped with a sampling tube. A Bioscan Triathler radioflow detector was placed inside the fumehood used with the HPLC system. For quantitative tritium measurements and wipe tests a Rackbeta LSC was used.

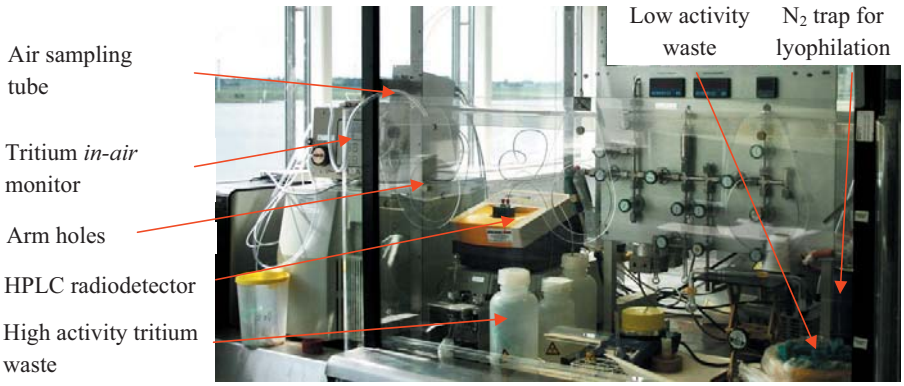


Figure 10. A picture of the laboratory installation, of the tritium manifold system with the required instruments for tritium detection. Arm holes were cut in the fumehood's front panel with slides for safe performance of gaseous tritium chemistry.

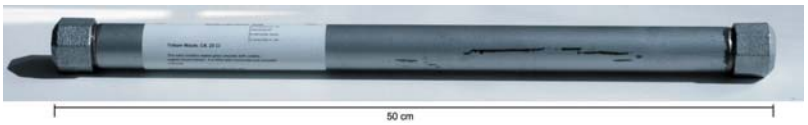
High amounts of radioactive tritium waste must be deposited according to the defined current law. Also, for radiation -safety and -protection reasons, described standard procedures for control of the tritium level of laboratory surfaces must be followed. In defined intervals and after each tritiation reaction, laboratory 'hot-spots' must be wipe tested and counted with liquid scintillation counting – the current Danish law has set a maximum limit of tritium to be 5000 Bq pr. cm^2 laboratory surface and 500 Bq pr. cm^2 skin surface.

In the Hevesy laboratory, tritium waste handling has been described in standard operating procedures, and the produced waste is divided into the following classes and handled accordingly;

Low amounts of tritium waste are gloves and utensils used in synthesis. This waste is double bagged and placed in white buckets for short time storage and deposited with the normal non-radioactive waste (less than 500 MBq per bag).

High amounts of tritium waste (more than 500 MBq per container), are further divided into two sub-classes; **1)** liquid HPLC waste and **2)** solid waste from tritium reactions. This waste is sealed in Kautex bottles as shown in figure 10 on the last page, and afterwards placed in yellow buckets, before it is sent to the Danish Decommissioning[25] for long term storage.

Figure 11. (above) Buckets for tritium waste. (left) Vessel and tubes for storage of radioactive by-products.



High amounts of volatile organic bound tritium waste, *e.g.* tritiated butane as a by-product from tritide reactions, are lyophilized into special glass vessels and stored in metal tubes containing absorbing materials (charcoal and vermiculite). See figure 11.

Schematised Principle of Tritiation Procedure

The Tritec RC manifold system is based on a tritium absorbed on uranium storage technology. When metallic uranium is exposed to dihydrogen, the gas is efficiently absorbed onto the surface as hydride (for tritium it is absorbed as uranium tritide (UT_3)). It is a very useful way for storage of tritium because the ditritium gas absorbs to a very low pressure at room temperature. The tritium gas is released from the storage bed by heating with a flame or heat gun to around 500°C (equals ca. 1 atm). A normal bed for storage of tritium contains 200 – 250 Ci of radioactivity.

A normal tritiation procedure with this uranium tritide technology is illustrated on figure 12.

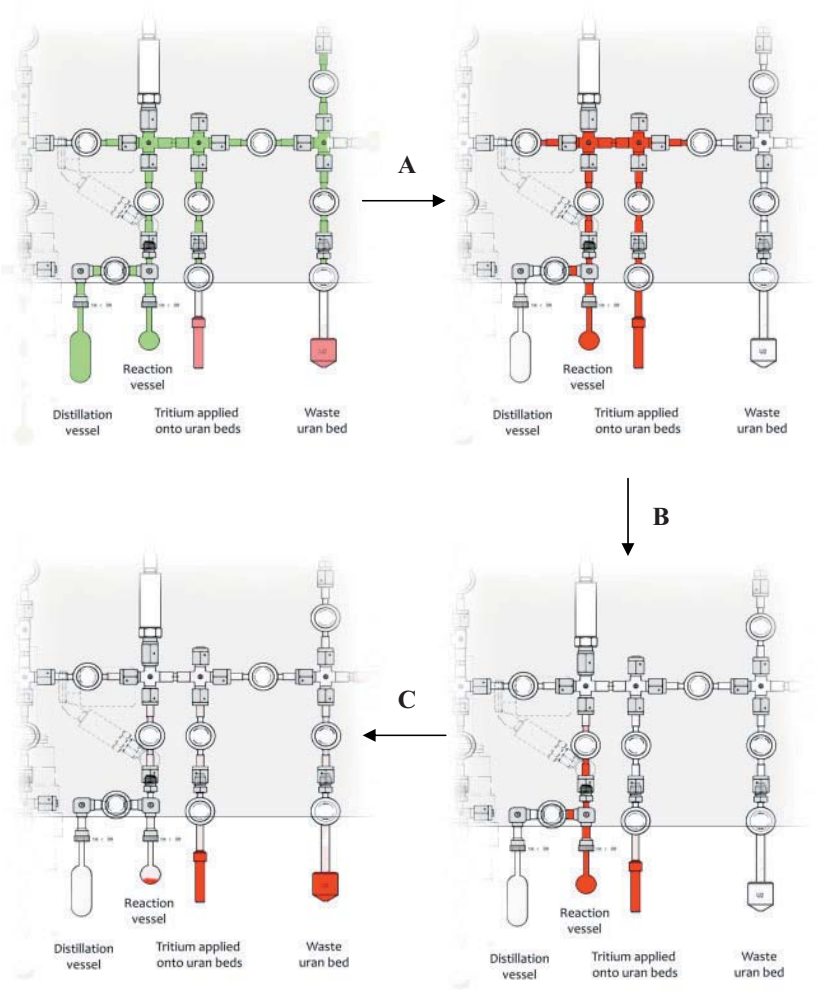


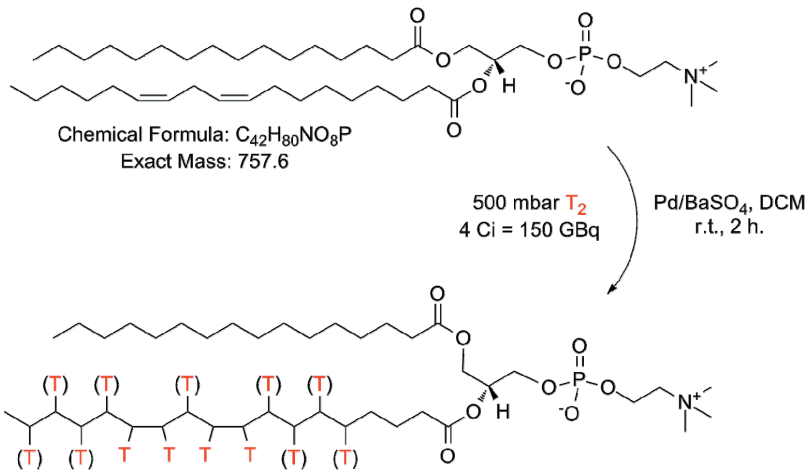
Figure 12. Illustration of a standard tritiation procedure (red = tritium). A) Tritium gas is released from the storage bed to the manifold tube system and into the reaction vessel. B) The valves are closed to the reaction and excess of tritium reabsorbed onto the storage bed. C) After end reaction the excess of tritium gas is absorbed onto a uranium waste bed.

Isotopic Scrambling Observed in Tritiation of Unsaturated Compounds

Heterogeneous Olefinic Tritiation of Unsaturated Phospholipids

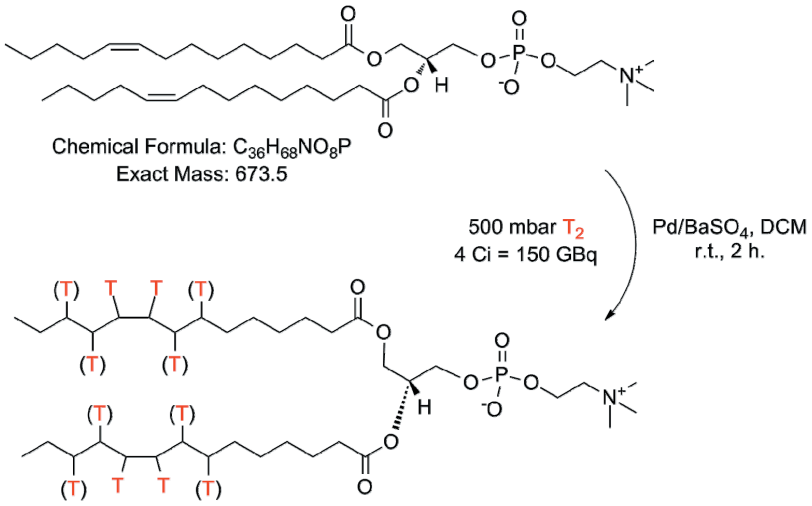
Homogeneous olefinic tritiation of unsaturated phospholipids were performed, in collaboration with research partners at the University of Copenhagen[26], and as a part of the bachelor project of Anna Pie Jakobsen Huda[27]. From a tritium chemist point of view, these experiments turned out to be excellent examples of the isotopic scrambling phenomena, regularly observed in olefinic tritiation reactions. The intension of this result section is to present a brief overview of the results from two reactions performed, as well as to give a short description of the mechanism behind isotopic scrambling.

Heterogeneous olefinic hydrogenation is a widely used and standard method for tritiation of unsaturated compounds. This is also mentioned in the section of standard tritiation methods described earlier. Tritium labelling of lipids by this method have been described in the literature[28-32]. The effect of reaction conditions on the incorporation of tritium into unsaturated compounds has also been investigated, and the mechanisms for isotopic scrambling have been proposed in one example from the literature[33]. The results, published, indicate the amount of tritium incorporated into unsaturated lipids, and is mainly based on the measured specific radioactivity. From theoretical calculations the number of tritium atoms inserted into the products was calculated. In the results presented here, the amount of tritium incorporation into unsaturated phospholipids was analysed by mass spectrometry.



Scheme 5. Schematic illustration of the tritium labelling of an unsaturated phospholipid (the first substrate; PLPC) using carrier-free tritium gas and a heterogeneous palladium catalyst ($Pd/BaSO_4$). The incorporation of tritium is distributed along the unsaturated carbon chain due to isotopic scrambling and double-bond migration throughout the carbon chain. Placement of the tritium atoms was not assigned in the tritiated product instead the possible positions are illustrated in parentheses.

Results, from the two tritium labelling reactions performed, are here presented as an illustrative example of isotopic scrambling. In scheme 5 (last page) and scheme 6, the overall standard hydrogenation reactions is illustrated, indicating the possible positions of the incorporated tritium.



Scheme 6. Illustration of the results from the tritium labelling of the second substrate; Δ9-cis-PC. In this case the unsaturated bonds are placed on the two different acyl chains.

Materials. All chemicals were used as received from commercial vendors. The unsaturated phospholipids 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (16:0 – 18:2) (PLPC) and 1,2-dimyristoleoyl-*sn*-glycero-3-phosphocholine (14:1 – 14:1) (Δ9-*cis*-PC) were acquired from Avanti® Polar Lipids, Inc. 5% palladium on barium sulfate was supplied by Sigma-Aldrich.

General tritium labelling procedure. A round bottomed reaction vessel (1 ml) was loaded with $Pd/BaSO_4$ (5.4 mg), the substrate [PLPC (16:0 – 18:2) (80 µl, 25 mg/ml in $CHCl_3$, 2 mg) or Δ9-*cis*-PC (14:1) (80 µl, 25 mg/ml in $CHCl_3$, 2 mg)] and DCM (920 µl). The vessel was then equipped with a magnetic stirbar and mounted onto the tritium manifold system. Subsequently the reaction mixture was cooled to -192°C with liquid nitrogen and evacuated to below $5*10^{-3}$ mbar. At this point a leak test was performed for ensuring a tight connection between flask and manifold. The flask was flushed with argon three times and left under an argon (ca. 1000 mbar). The reaction mixture was then allowed to warm to room temperature while stirring in 5 minutes for deoxygenising of solvent. The mixture was then recooled with liquid nitrogen and evacuated to below $5*10^{-3}$ mbar, before carrier-free tritium gas (T_2) was released into the reaction vessel to a pressure of 500 mbar (the radioactivity amounts used was calculated to be approximately 4 Ci). The reaction was allowed to warm to room temperature then left with stirring for 2 hours at room temperature.

After reaction, the vessel was unmounted from the manifold and the catalyst was filtered off through a 1.2 µm Whatman GF/C filter. The filtrate was lyophilized twice with addition of MeOH (2ml) for the removal of labile tritium and subsequently the product was subjected to HPLC for purification.

Preparative HPLC was performed on a Waters Breeze system. Pump: Waters 1525, Binary Pump. UV Detector**:** Waters 2787, Dual λ Absorbance Detector. Column: Phenomenex Jupiter C-4 column at ambient temperature (20°C). Mobile phase: A (5% MeCN, 0.1% TFA) and B (99.9% MeCN, 0.1% TFA). Gradient: 0-2 min (20%B), 2-10 min. (20%B to 100%B), 10-30 min. (100%B), 30-32 min. (100%B to 20%B), 32-35 min. (20%B). Flow: 1 ml/min.

[T]^{n}-1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphocholine (16:0 – 18:0) (PSPC): The radioactive products were collected from 14-19 minutes, analysed by LSC to yield total radioactivity of 9.0 GBq (243 mCi) and with mass spectrometry for investigation of tritium incorporation. Mass spectrum of products showed a distribution of several tritiated products. The range of tritium atoms incorporated into the phospholipid is illustrated by mass spectrometry in figure 13 (from 1 to 22 T).

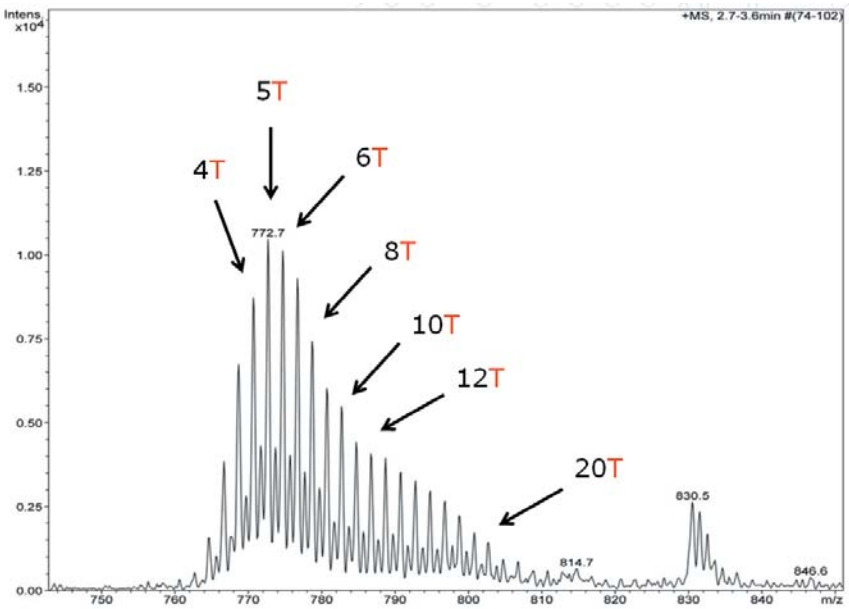


Figure 13. Mass spectrum of tritiated phospholipid (16:0 – 18:0) showing the distribution of products obtained by heterogeneous tritiation of PLPC (16:0 – 18:2). The amount of T-atom incorporations into the carbon-chain is estimated to be between 1 and 20, giving a calculated specific activity from 28.8 – 576 Ci/mmol.

[T]^{n}-1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (14:0 – 14:0) (DMPC): The radioactive products were collected from 14-22 minutes. Products were analysed by MS for the analysis of tritium distribution, as illustrated on figure 14 (from 4 to 22-24 T).

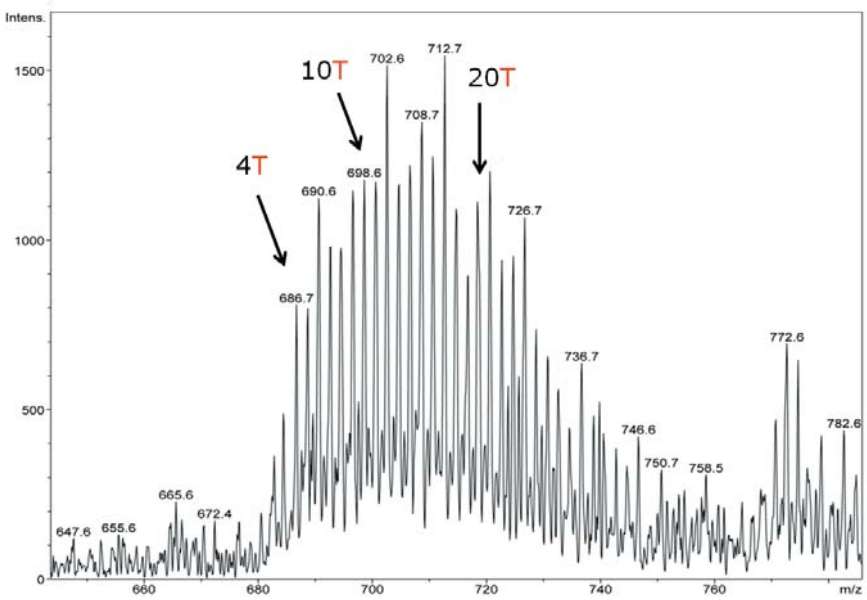
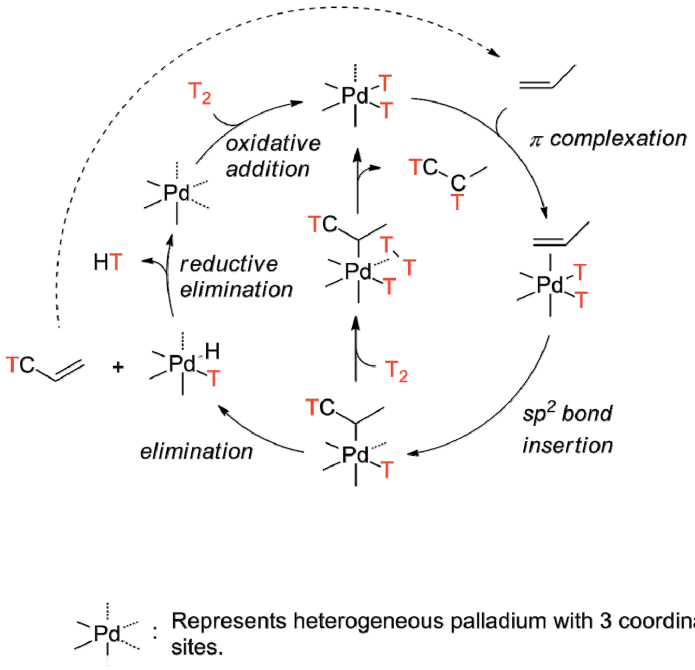


Figure 14. Mass spectrum of the tritiated phospholipid (14:0 – 14:0) showing the distribution of products obtained by heterogeneous tritiation of Δ9-*cis*-PC (14:1). The amount of T-atom incorporations into the carbon-chain is estimated to be between 4 and 22-24, giving an approximately specific activity from 115.2 – 691.2 Ci/mmol.

Conclusion. The heterogeneous olefinic tritiation reactions of unsaturated phospholipids performed here are illustrative examples of the isotopic scrambling phenomena. This will occur if elimination of a new C=C bond is favoured in contrast to formation of a new C-T bond. A proposed mechanism for this T-H scrambling is illustrated in scheme 7 (on next page). This mechanism presented here is simplified since many possible mechanisms can be imagined by the use of heterogeneous palladium. The proposed mechanism presented here is illustrated with 3 free bonding sites on palladium. These 3 vacant bond sites are imagined to be placed on the particular palladium surface and the occupied sites to bind with the either the support or heterogeneous palladium.

Numerous possible products can be imagined from this hydrogenation reaction. If the elimination step in the mechanism is favoured, the newly formed olefin can re-enter into the catalytic cycle. The tritium atoms will in this tritiation wander downwards the unsaturated lipid carbon chain. For this particular substrate the formation of a conjugated sp^2 bond could be favored and consequently result in many tritium atoms being incorporated.



Scheme 7. Simplified mechanism of isotopically tritium scrambling. The low partial pressure of tritium gas in this reaction is imagined to favour elimation of a new olefin over saturation of the carbon-carbon bond.

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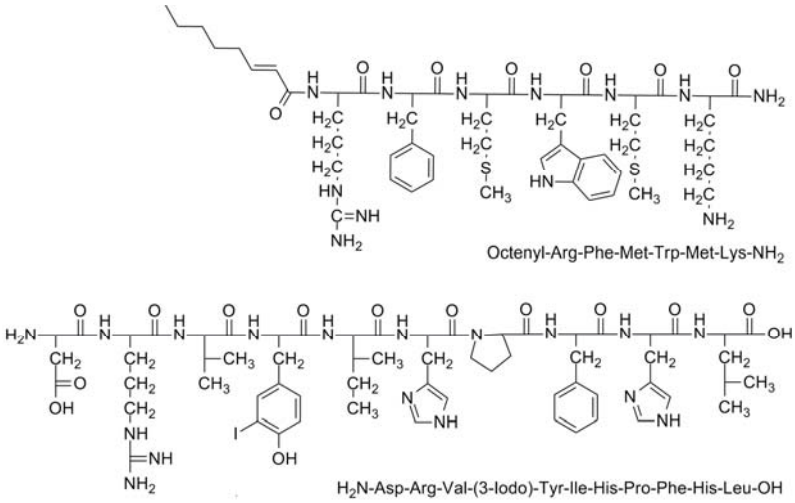
Part II.

Development of New Tritium Labelling Methods for Peptides



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Front page: Illustration of the chemical structures of two synthesised peptide substrates for development of new homogeneous hydrogenation chemistry. The first is a conjugated enkephalin substrate for investigation of the olefinic hydrogenation reaction. The second is an iodinated angiotensin for investigation of dehydrohalogenation reaction.

Short Perspective, Objectives and Aims

There is an enormous and increasing market in the field of therapeutic peptides and proteins and the medicinal industry have turned their priorities towards development of these biopharmaceutical drugs. In an era[1] of 'new' synthetic polymeric formulations and bio-conjugated drugs the demand for clinical studies from the authorities is naturally increasing for development of safe therapeutics.

The main objective of these graduate studies was the development of new tritium labelling methods for peptides and/or proteins. The current first choice and preferred tritium labelling methods for peptides and proteins involve either tritiodehalogenation of pre-halogenated peptides or tritio-acyl conjugation onto nucleophilic amino- or thiol- functional groups.

Other methods have been presented in the literature but often these are more 'specialised' and some are not generally applicable for labelling of newly developed drug candidates.

For small peptides (up to approximately 30 AA) heterogeneous palladium tritiodehalogenation is feasible but problems have shown to occur when larger substrates are subjected to heterogenous catalysts and supports[2]. Whether it is due to clotting or sticking of the substrates to the solid catalyst or inaccessibility at the active site on the peptide or protein for the catalyst is undetermined.

Within the last 5-10 years, a few research groups have presented methods for derivatising synthetic polypeptides and proteins with homogenous palladium coupling techniques (*e.g.* Sonogashira, Suzuki) and with some degree of success[3]. Considering the chemical properties of these substrates; high molecular weights, diversity of functional groups and fragile tertiary structures play important roles and also dictates the limitations, for allowable reactions conditions.

Therefore, organometallic reactions on peptide and protein substrates must be carried out under mild conditions; preferable in a (buffered) aqueous medium, at temperatures to avoid denaturation and neutral to slightly acidic pH. For proteins, often salts and surfactants are necessary to maintain proteins in their native state[4].

There is a need for new and efficient labelling methods for peptides and proteins - this is why we initiated an investigation for the use of homogenous catalysis for the tritiodeiodination of peptide substrates.

Background

Perspective on Therapeutic Peptides and Proteins

Peptides and proteins are responsible for carrying out many important biological processes in living organisms. These biological macromolecules built of poly-amino acids – possess an enormous potential as pharmaceutical drugs. The market for proteins and peptides as biopharmaceutical drugs have been increasing for the last decade, which have also resulted in an expansion of the field of marketed synthetic therapeutic peptides[5].

Insulin was the first protein to be isolated and administered therapeutically and it remains today as a block buster biopharmaceutical drug for treatment of diabetes[6]. The discovery of insulin by Banting and Best[7] in 1921 is perhaps one of the greatest discoveries in the history of medicine. This landmark discovery was thus also awarded the Nobel prize in physiology or medicine in 1923[8], and this hormone is perhaps the only single molecule known to be subject to three Nobel prizes in different scientific disciplines. Frederick Sanger in Chemistry (1958) for his work structure determination of proteins especially insulin [9, 10] and later in Dorothy C. Hodgkins (in 1964) for her work on three dimensional x-ray structures on macromolecules and insulin [11, 12].

More than 60 therapeutic peptides with less than 50 amino acids have found their way onto the pharmaceutical market for applications towards a variety of diseases *e.g.* cancer, diabetes, cardiovascular/CNS diseases, infections, inflammation and obesity[5]. Within the total pharmaceutical market, peptides and proteins reached $28 billion in 2000. Regarding the market for synthetic peptides alone, in the time-span from 2003 to 2005 the market increased from €5.3 billion to €8 billion and it is estimated to reach €11.5 billion in 2013[5, 13].

Compared to small molecules - peptides and proteins have several advantages which include high affinity and specificity against their molecular targets. They bear unique three dimensional characteristics with chemical functionalities and properties ‘designed’ for a biological function. *In-vivo* therapeutic peptides and proteins can be designed to have a relatively low immunogenicity as they are build of the natural building blocks; amino acids[13].

In contrast, the main drawback of peptides and proteins as pharmaceutical drugs remains the low bioavailability in orally dosed forms, low metabolic stability *in-vivo* and often high manufacturing costs compared to the small molecule therapeutica[13].

Bioavailability and biodistribution of peptides are mainly determined by their physiochemical properties; therefore peptides composed of natural amino acids are generally considered poor drug candidates (as orally dosed drugs). According to the ‘Rules of Five’ describing the ‘*drug-like-ness*’ of a new molecular entities (NME) formulated by Christopher Lipinski[14, 15], and the further appliance of these rules on

peptides support the poor bioavailability when dosed orally[16]. Out of several reasons, these biopharmaceuticals are simply too large molecules and have too many hydrogen bond donating and accepting groups to be absorbed from the *intestinal mucosal* membranes. For these reasons, until a few years ago, peptides and proteins were administered only by injection, either by subcutaneous, intramuscular or intravenous injection routes. After injection and the entrance into the body-fluids these then meets another challenge. Numerous of proteolytic enzymes with high selectivity against peptide bond cleavage are found *in-vivo*[17]. These enzymes are responsible for the often shorter half-lifes and the low metabolic stabilites of peptide drugs based on natural amino acids.

To improve the physiochemical properties and the *in-vivo* stability of poly-peptides, drug discovery programs often include the use of unnatural amino acid residues and/or synthetic post-production modification of the polymer chain [18, 19]. The scope of biopharmaceuticals (polymeric drugs) based on these modifications is without boundaries. The modification of a natural occurring peptide or protein raised a demand from the regulatory authorities for thoroughly performed clinical trials. An in-depth investigation of the pharmacokinetic and dynamic (PK/PD), as well as of the distribution, metabolism and excretion properties (DME) must be undertaken for these new molecular entities (NME)[20]. A (by now) standard and FDA approved modification of peptides and proteins is PEGylation and several of these modified drugs are currently used in clinics[1]. The synthesis of "new" well designed types of pharmaceuticals, together with the more stringent demands from the regulatory authorities (*e.g.* FDA and EMEA), also gives rise to a demand for the development of new and efficient radioactive labelling techniques of NMEs.

When the traditional and non-modified peptide or protein drugs (*e.g.* Insulin) were approved as therapeutically used drugs – those could be argued to behave as the biomolecules already a part of the human-body. This argumentation resulted in more relaxed restrictions for the performed clinical trials from authorities.

Chemical Synthesis of Peptides

The structural diversity of the natural amino acids and with introduction of unnatural synthetic building blocks gives almost unlimited possible poly-peptide sequences. Peptides can be prepared (synthetically) by, recombinant methods, modification of isolated natural products, or lastly chemical synthesis in either solution or on solid support. It is possible to synthesise peptide therapeutics in multi kilogram scales and it is done in production environments. In the search for peptide drug candidates, synthesis is often performed on solid support and many techniques for synthesis of large combinatorial peptide libraries have been developed[21]. Total synthesis of peptides is a very well-described and still developing science [22].

In this thesis, Solid Phase Peptide Synthesis (SPPS) have been applied for the synthesis of two peptide substrates. These substrates were used as model compounds for developing new deuterium and tritium labelling techniques. A brief introduction

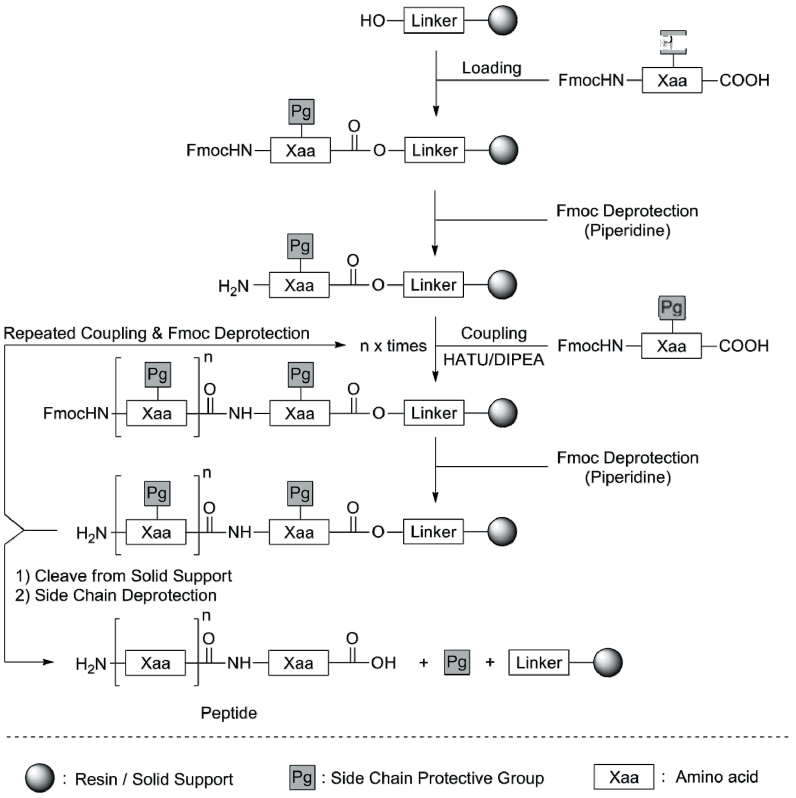
to the history and development of the chemical synthesis of peptides is therefore presented here.

The first description of the peptide-bond synthesis of dates back to around year 1900 by Hermann Emil Fischer[23]. Fischer was limited to the chemistry of simple unprotected amino acids in solutions which had its limitations. Since then an evolution has happened on the development, analysis and use of peptides. Later on, the introduction of functional group protection on amino acids, made it possible for in-solution synthesis of several natural peptides[24-26].

The major breakthrough for peptide synthesis was the invention of chemical synthesis of peptides on functionalised solid matrixes by Bruce Merrifield in 1963[27]. With his article, he demonstrates the synthesis of a tetrapeptide on insoluble polymer beads of styrene and divinylbenzene. This invention has indeed revolutionised the area of peptide synthesis and Merrifield was thus recognised with the Nobel prize in 1984 for his work[28].

The basic strategy of SPPS is repeated amino (NH_2-) and side chain- protected amino acid couplings onto a functionalised insoluble solid support. The main advantage of chemistry on a solid support is easy removal of excess used coupling reagents by filtration and washing.

Today, numerous solid matrixes, coupling strategies and amino acid protections schemes have been developed[29]. For a basic overview of SPPS with use of the Fmoc methodology (as applied for synthesis of the radiolabelling substrates) please refer to **scheme 1** (next page).



Scheme 1. General overview of solid phase peptide synthesis using the Fmoc methodology, repeated HATU/DIPEA couplings and a hydroxyl-group functionalized solid support linker.

Stepwise; (a standard sequence for peptide synthesis) begins with anchoring the first amino acid at the carboxyl group onto the solid support with a cleavable linker (loading). After the loading sequential (selective) N-deprotections and amino acid couplings are performed for construction of the peptide. After iterative FmocNH-deprotections and couplings, the peptide is cleaved from the support by conditions determined by the linker.

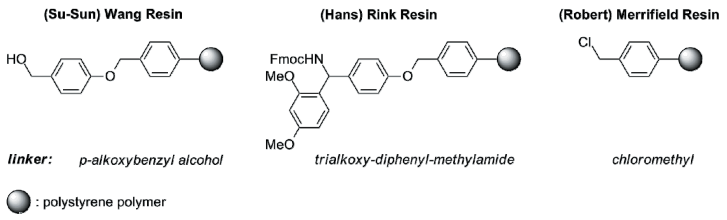


Figure 1. Linkers of the Wang, Rink and Marrifield resins.

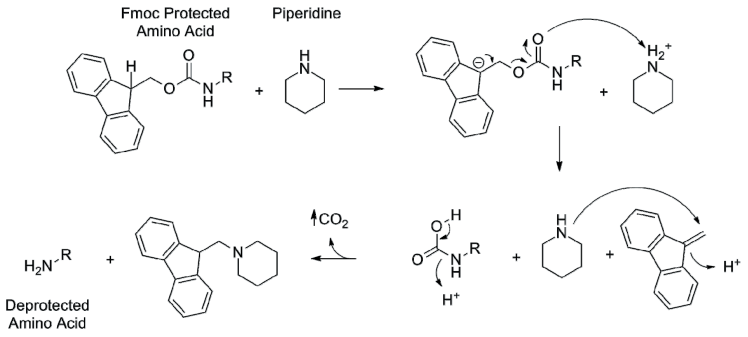
Three resins with functionalised linkers are illustrated in **figure 1** (last page). In this thesis two types of resins have been used for the synthesis of the peptide substrates. For synthesis of Angiotensin-I the Wang resin was applied (please refer to the experimental section in appendix for details of this peptide synthesis). This resin is functionalised with a 4-benzyloxybenzyl alcohol linker and when the poly-peptide are cleaved from this resin the C-terminal becomes a carboxylic acid[30]. The modified enkephaline substrate, which was also synthesised, has a C-terminal amide function. For this the Rink amide resin[31], functionalised with a trialkoxy-diphenyl-methyl Fmoc-amine, was applied (details about this peptide synthesis are described in the result section of this part). For comparison, the Merrifield resin, functionalised with a chloromethyl linker, originally applied and developed by for the first poly-peptide synthesis[27], is also depicted on the figure. (The synthesis of the peptide substrates are described in greater detail in the result section).

In the end of a synthesis - when the peptide is cleaved form the resin - the amino acid side group protections can also be cleaved. It depends on the later application of the peptide, if the side-chain protective groups are wanted cleaved or kept if these are needed in subsequent solution based reactions. Protection schemes and a more labile linker can be applied for this.

There are two different N-protecting group methodologies in SPPS, fluoren-9-ylmethoxycarbonyl (Fmoc) and *tert.*-butoxycarbonyl (Boc). The Boc strategy introduced by Merrifield takes advantages of the difference in lability of the N-protective group and the side chain protective groups, which are cleaved by trifluoroacetic acid (TFA) and hydrogen fluoride (HF) respectively[32].

The use of hydrogen fluoride (HF) as the cleavage reagent, is not without any risks and can cause severe chemical burns, and HF chemistry also require special equipment. This is why peptide chemists have now phased out Boc chemistry and in replacement use Fmoc as the amino protective group. This revolutionary amino protection group was first described by Louis Carpino in 1970[33] as a new base labile amino protection group for use in organic syntheses[34, 35]. The mechanism for Fmoc deprotection is illustrated in scheme 2.

In the work of this thesis, Fmoc chemistry have been applied[29]. In general the base (piperidine) facilitates the cleavage by elimination of dibenzofulvene followed by an irreversible decarboxylation, leaving the deblocked/unprotected free amine.



Scheme 2. Mechanism for the Fmoc deprotection of amino acids in SPPS.

In the general peptide synthesis, the peptide bond is formed by the reaction of an activated carboxylic acid to a free deblocked amine. Many activation reagents have been developed and shown to be applicable for this reaction. In the beginning of the SPPS era, carbodiimide couplings were generally applied. Later on, highly efficient coupling reagents have been developed. In figure 2 (next page), a very limited selection of these coupling reagents are illustrated. In this thesis the HATU coupling reagent have been utilized, together with the non-nucleophilic base DIPEA. The addition of HOBt or HOAt, have been shown to ensure, the optical integrity of the stereogenic center at the C-terminal of the activated carboxylic acid in the coupling reaction[36].

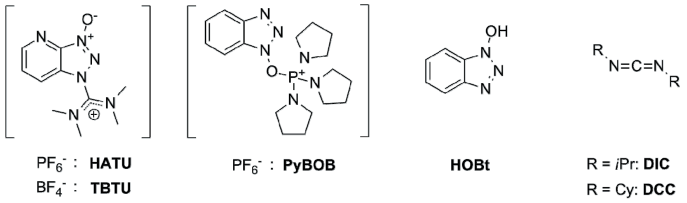
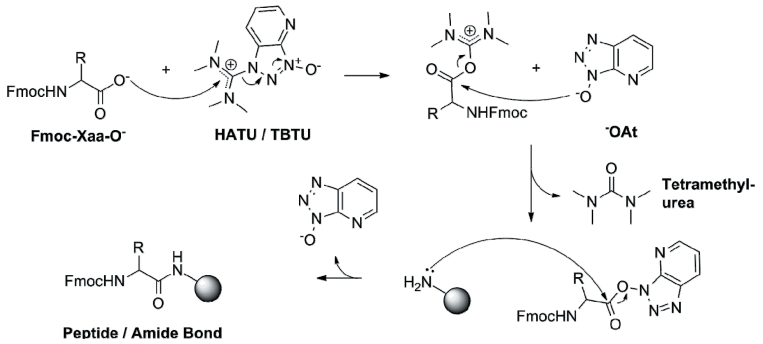


Figure 2. A selection of commonly used coupling reagents.

The mechanism for the HATU/DIPEA peptide coupling reaction are proposed to proceed as shown on **scheme 3**.



Scheme 3. Coupling mechanism for the formation of a peptide/amide bond with HATU or TBTU and DIPEA.

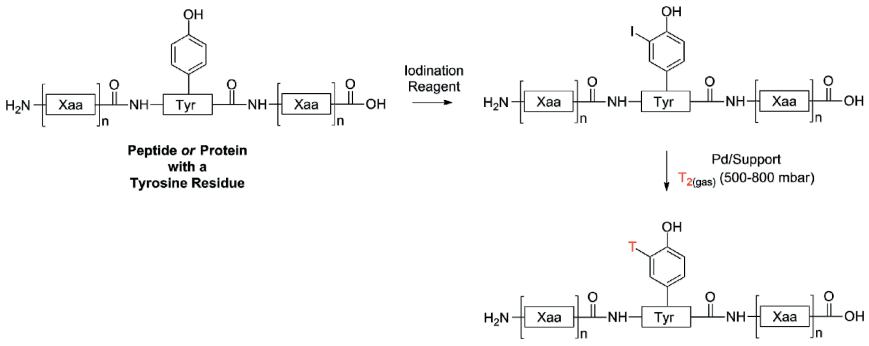
Part II. Development of New Tritium Labelling Methods for Peptides.

Current Methods for Tritium Labelling of Peptides and Proteins

In this section the current methods for tritium labelling of peptides are given as a short overview. Several approaches have been attempted for this radiolabelling of poly-peptides, some methodologies more applicable than others.

Tritiodeiodination

An extensively used tritiation method for general labelling of new molecular entities (NMEs) in particular is the tritiodehalogenation reaction (please refer to Part I of this thesis for an overview of general tritium chemistry). This tritiation method has also been used for peptides in several publications [37-46]. The method has advantages in the labelling of peptides and proteins with a tyrosine residue included into the peptide backbone, because these residues are readily iodinated using organic or enzymatic iodinations[47-49]. Alternatively, an iodinated tyrosine can be incorporated in the synthesis of the peptide.



Scheme 4. A general tritiodeiodination reaction on peptide or proteins in two chemical steps.

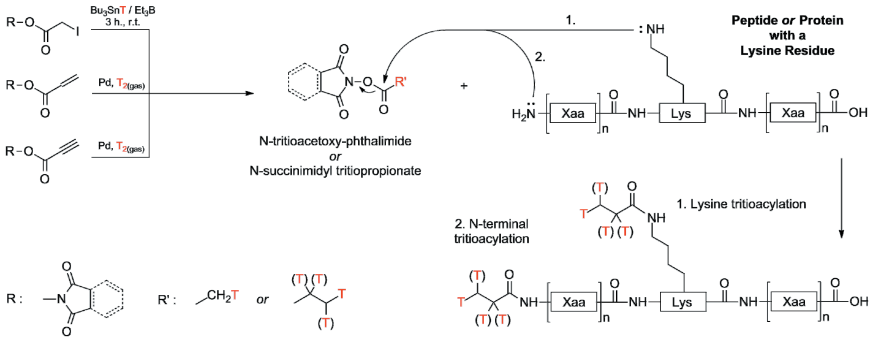
In **scheme 4**, a general overview of tritiodeiodination on peptides is outlined. First step is iodination of a tyrosine residue with the use of an electrophilic iodide source, secondly the actual tritiodeiodination is performed using gaseous tritium and a heterogenous palladium catalyst.

Tritioacylation

The commercial availability of tritioacylation reagents, such as [^{3}H]acetic anhydride have made this method for labelling of peptides and, in particular, proteins one of the preferred choices[50].

The introduction of N-tritioacetoxyphtalimide and N-succimidyl 2,3-[^{3}H]propionate with a high specific activity have made these reagents favourable. They eliminate some of the disadvances from the smaller [^{3}H]acetic anhydride, such as its volatility and unavoidable loss of radioactivity (50%) when this reagent is applied.

Tritioacylation reagents are readily available by synthesis which is shown on scheme 5. The *N*-tritioacetoxy-phthalimide reagent has been prepared by radical tritiodehalogenation of the iodinated precursor[51] and *N*-succinimidyl tritiopropionate can be prepared, either by gaseous reduction of an unsaturated propenyl/propynyl acid followed by coupling to *N*-hydroxy succinimide or by the direct reduction of the already coupled succinimidyl[52], shown in **scheme 5**.



Scheme 5. A general tritio- acetylation/-propionylation labelling of peptides and proteins. The labelling reagent *N*-tritioacetoxy-phtalimide can be prepared using radical tritiodeiodination with Bu_3SnT and *N*-succimidyl tritiopropionylate by tritiation of the corresponding alkene/alkyne.

Tritiation by conjugation onto peptides and proteins with these reagents can be performed onto free amino (lysine residues or *N*-terminal) and thiol functionalities but not hydroxyl groups. Labelling can be performed in buffered solutions at pH 7-8 and at conditions from 0^oC to room temperature in a wide variety of solvents (MeCN, DMSO, MeOH, dioxane and water) [52-54].

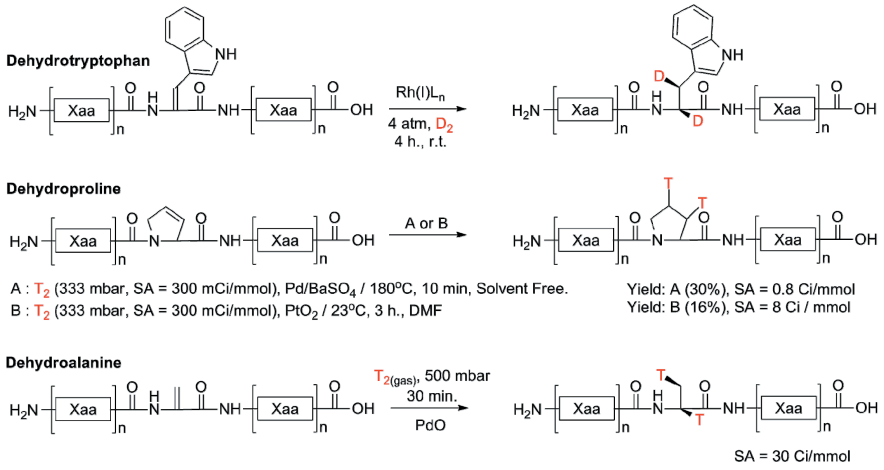
Although this method is preferred as a tritiation method of proteins, the conjugation interferes with the biological properties of the labelled molecule. Especially if a small peptide substrate is labelled, this conjugation could be a significant structural change and have a great impact on the peptide affinity towards its target. At physiological pH the lysine residues are protonated and conjugation will prevent this.

Olefinic Hydrogenation using Homo- and Hetero-geneous Catalysts

The incorporation of tritium into peptides has also been reported by olefin hydrogenation of unsaturated amino acids. A prerequisite for this method to work is introduction of an unsaturated amino acid residue into the backbone of the peptide. Several more or less successful methods have been described; an exotic enzymatic 2',3'-oxidase of tryptophan have been reported [55], otherwise the perhaps most accessible way is with the use of an unsaturated amino acid in SPPS [56-60].

On **scheme 6**, three different labelling reactions are illustrated (Please refer to Part I of this thesis for a more general overview of different hydrogenation methods). A standard olefinic hydrogenation uses either a heterogeneous palladium or a homogenous rhodium (Wilkinson's) catalyst in an organic solvent and tritium gas.

In the examples in **scheme 6** two different methods have been highlighted for tritiation of dehydroproline. The first uses Spillover Tritium (ST) with a solid catalyst and the second a heterogeneous catalyst in solution. A large difference in specific activity is noted in these two reactions. It must be noted that there is no general trend in which method gives the highest specific activity[60].



Scheme 6. Literature examples of deuterium and tritium labelling of unsaturated amino-acid residues of tryptophan, proline and alanine[55, 57, 60]. Three hydrogenation methods are illustrated here, homogeneous with Rh(I), heterogeneous with PdO and solvent-free high temperature H/T exchange.

It is also important to notice that the tritiation of dehydrotryptophan and dehydroalanine in the reactions above is performed at the stereogenic center of the amino acid residue. This is why these two methods, in my opinion, are in general not applicable in labelling of peptides and proteins. A subsequent necessary resolution of the two enantiomeric generated peptides could be imagined as a doubtful procedure.

High-Temperature Solid-State Catalytic Isotope Exchange

The last method, which will be mentioned in this section, for tritium labelling of amino acid residues in peptides, is the use of Spillover Tritium (ST) in solvent free reactions[61]. This labelling technique is performed in solid state at high temperature with the use of a low pressure of tritium gas.

In general the peptide/protein is evaporated onto a solid support *e.g.* aluminium oxide, and a solid hydrogenation catalyst such as palladium, rhodium or platinum is

added[62]. When this solid mixture is heated to 100°C - 200°C under an atmosphere of hydrogen gas - isotopic exchange (H/T exchange) will occur randomly and with high labelling efficiency (SA up to 2000 Ci/mmol ≈ 70 T/mmol).

The High Temperature Solid-State Catalytic Isotope Exchange (HSCIE) labelling method has been employed for proteins isolated from extremophile microorganisms[63] with only minor loss of enzymatic activity when labelled at temperatures below 120 °C [64]. This method is limited to a few published examples because of the known loss of amino-acid chirality and denaturation at elevated temperatures[62, 65].

Homogenous Catalysis and Peptides

A brief introduction to the concept of homogenous catalysis, followed by an overview of applications with peptides and proteins is described in this section.

The field of homogenous catalysis is enormous and is thus the subject of extensive research in chemistry groups, and excellent research articles, as well as, many reviews on this topic, have been published[66, 67].

Palladium and Phosphorus Ligands in Organocatalysis

Some of the most popular catalytic C-C bond formation reactions involves the use of palladium(0) complexed by phosphorous ligands. Palladium (0) is a d^{10} element and most of the stable and isolable palladium(0) complexes follow the 18- of 16-electron rule[68] (with equivalent 4 or 3 lone-pair donating ligands).

Palladium(0) complexes (*e.g. tetrakis*(triphenylphosphine)palladium(0)) tend to exist as a coordinatively saturated 18 electron tetrahedral d^{10} complex, but depending on the ligand, they readily dissociate into the reactive and coordinatively unsaturated 16- or less-electron species. Two properties of the coordinating ligands are primarily considered to determine the reactivity of the catalyst – the electronic and the steric properties of the ligand. Before the 1970's electronic properties, of ligands, were general considered to dictate the reactivity of the catalyst, until, Chadwick Tolman in the 1970's investigated phosphine ligand steric properties in depth. Quantitative ligand dissociation of nickel(0) carbonyl substituents were correlated with measured phosphine cone angles[68-71]. The experimental values from these experiments and the measured cone angles are illustrated on **figure 3,** and as it appears, the more bulky phosphines (with large cone angles) replace less carbonyl ligands from $Ni(CO)_4$. For more precise determination of the phosphine cone angles calculations by mathematical/computational methods or estimation by x-ray structural data is today the method of choice[69].

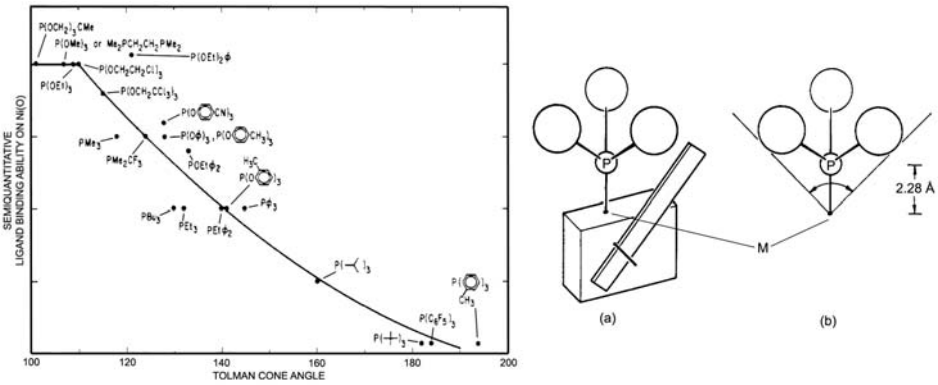
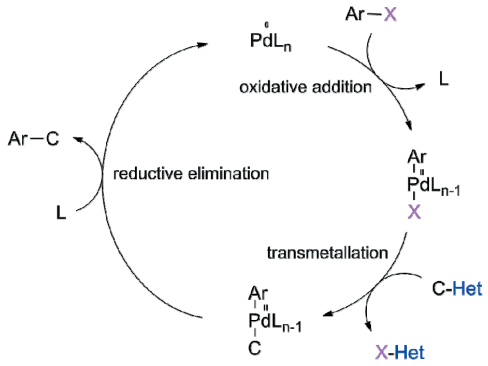


Figure 3. (*Left*) Plot (reproduced from the litterature[68]) of the experimentally measured phosphorous Tolman cone angle (Θ) versus the ligands binding ability towards Ni(0). (*Right*) (a) Original device by Tolman, for experimentally measurement of the Tolman angle of phosphine ligands[72].

The extent of ligand dissociation in phosphine palladium complexes increases in the order PMe_3 < PMe_2Ph < $PMePh_2$ < PEt_3 < PPh_3 < $P(iPr)_3$ < PCy_3 < $PPh(tBu)_2$ < $P(tBu)_3$. In general phosphines with large cone angles tend to dissociate to a larger extent than the phosphines with smaller cone angles. This gives rise to low ligated and reactive 14 e- and 16 e- species. Hartwig et al. have performed investigations of the properties of low-ligated palladium species with bulky-phosphines. It was found that these are very efficient for some reaction types (*e.g.* for the cross-coupling of aryl-chlorides which are normally inactive in these reactions)[73-77]. When the tri.-*tert.*-butylphosphine ligand (a bulky phosphine with a cone angle of 182°) is employed with palladium, it forms a *bis*-ligated 14 electron palladium species. This ligand has been utilized and found useful for cross coupling reactions. Because of the low ligated palladium an increased reactivity is observed and often reactions can be performed at room temperature. Cross-coupling reactions with $P(tBu)_3$ have been pioneered by Gregory Fu et al[78-83]. The increased reactivity / reaction rate is expected to be a consequence of a favoured oxidative addition step in the catalytic cycle (a general catalytic palladium cycle is shown in **scheme 7**).



Scheme 7. General accepted cross coupling reaction mechanism. X = Halogen, OTf. L = Phosphine ligand. Het = Boron, Silicon, Tin ect.

The cross coupling catalytic cycle consists of three important individual steps. First the oxidative addition of palladium(0) across an aryl-X bond for the formation of a palladium(II) species. The X stated in the scheme is the leaving group, and the kinetics for this reaction depends on the element. Approximate orders of reactivity of varying leaving groups are as follows: I > OTf > Br > Cl >> F. Following step of the cycle is the transmetallation step. It has been proven that many C-heteroatom compounds are feasible for this.

Important cross-coupling reactions include: The **Suzuki** coupling with use of C-boronic acids[84], the **Stille** coupling with the use of C-alkyltin compounds[85] and the **Hiyama** coupling with use of C-silicon[86-88].

Palladium(0) Catalysed Reactions for Conjugation of Peptides and Proteins

In the past decade palladium catalysed reactions for site specific conjugation of peptides and proteins have gained more interest. Although the reported examples are sparse; Suzuki, Sonogashira and Heck type cross coupling of iodinated phenylalanine and tyrosine residues have been presented in the literature.

Although proteins can be functionalised they are still difficult to functionalise by organic reactions. The large steric hindrances from neighboring amino acids residues limit access to the reaction site within the protein[89]. This could result in prolonged reaction times or no availability at the reaction site.

The pioneering example of protein bioconjugation, by an aqueous Sonogashira coupling, were reported by Schmidtchen and Dibowski in 1998[90]. In their paper they present the use of water soluble phosphines as the palladium ligand.

Subsequent examples include; Arylation of diiodotyrosine[91], one-pot borylation and homo-coupling of iodinated tyrosines[92] and functionalization of *p*-iodo-phenylalanine of proteins[93] (**figure 4**).

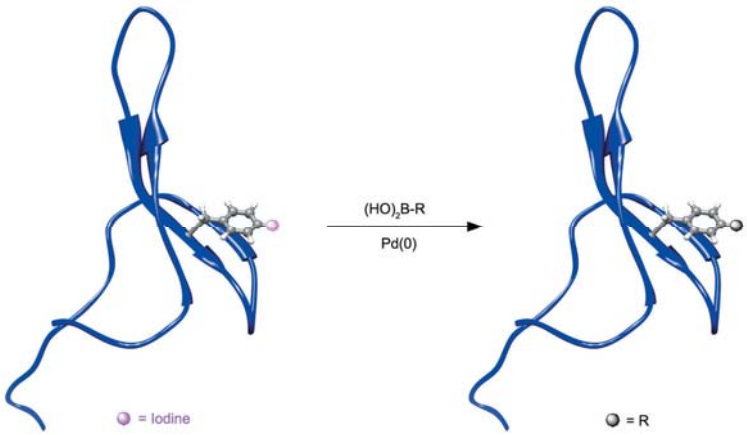


Figure 4. Suzuki-Miyaura cross coupling for bioconjugation of p-iodo-phenylalanine in the WW domain of the Pin1 protein (Time 20 h., Temp. 20 °C).[93] The illustration have been generated from a PDB file (1I8H.pdb) using UCSF Chimera as the software[94].

Other important examples include the Heck and Sonogashira coupling of alkene/alkyne derivatives onto iodinated amino acids[95, 96]. These few publications are outstanding examples, demonstrating homogenous catalysis with palladium(0)-phosphines on peptides and proteins as the diversity of amino acid functionalities such as hydroxyl, carboxylic acid, amine, thiol could be imagined to 'poison' and deactivate the catalyst.

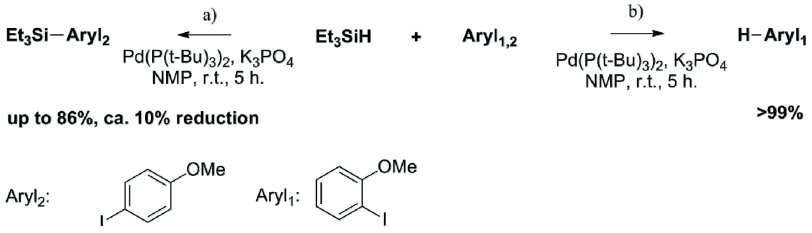
Silicon in Cross-Coupling Reactions

Silanes (R_4Si) and hydrosilanes ($R_{4-n}SiH_n$) have many applications in synthetic organic chemistry. They are used as reagents in palladium cross coupling reactions, as radical and non-radical reducing agents. Their strong advantages are manifested in combination with transition state metals, where they function as both reducing and C-Si and C-C bond forming reagents. In combination with a radical initiator (*e.g.* AIBN) they can also function as radical based dehalogenation reagents[97].

The silicon based C-C cross coupling reaction is a well known reaction discovered by Tamejiro Hiyama[88] in 1988[86, 87]. This reaction uses fluoride as a silicon activation reagent for the formation of a penta-coordinate silicate ion which is much more active in the cross coupling reaction[98]. Silicon cross coupling reactions have since been well studied and found many useful applications for C-C bond formation. One of the pioneers is Scott Denmark, who, as an example, has described the use of silanols in various palladium coupling reactions[99]. Also trialkoxy-silicon compounds have been used in coupling reactions, with[78] and without[100] the use of phosphine ligands.

Hydrosilanes which are demonstrated as relevant hydrogen labelling reagents later in this thesis, have also found many other useful applications. In combination with a suitable and homogeneous hydrogenation metal catalyst they can be employed as reducing agents for *e.g.* hydrosilylation of unsaturated C-C bonds[101].

Hydrosilanes have also been demonstrated as suitable carbon silicon bond formation (C-Si) reagents in palladium cross coupling reactions. Especially in recent years a number of publications have emerged on this topic. Triethylsilane has *e.g.* been used in a triethylsilyl-aryl couplings with the use of PtO_2[102] or palladium catalysts[103, 104] and aryl-iodide as the substrate.



Scheme 8. Reported method for a) Carbon Silicon bond formation[103] or b) reduction of aryl-iodides[105]. The same catalyst and reaction conditions form the diffent products. As reported the different reaction paths is determined by the substrate in these reactions.

On the other hand, triethylsilane has also been reported as selective hydrodehalogenation reagents in the same reaction, but with slightly different substrates, with homogeneous palladium(0) catalysts. Depending upon the choice of

phosphine ligand, substrate and reaction conditions, the hydrosilanes therefore either functionalise or hydrodehalogenate the substrate as shown on **scheme 8**. The selectivity in the two reactions schemed above are argued to be an effect of steric hindrance. While the *ortho*-iodoanisole forms only the reduced product, the *para*-iodoanisole substrate was reported gives up to 86% silylation and approximately 10% reduction. At the end of the result section, two similar reactions with the same reaction conditions, but without the use of base are shown.

Recent publications report the use of triethylsilane as reducing reagents (for the substrates Ar-OTf and Ar-I) with the use of palladium and bulky phosphine ligands [105-107].

As described in the last section, the application of Si-H activation chemistry is a very useful tool for the formation of the synthetically useful C-Si bond. In addition, the hydridic character of the hydrosilane makes these useful reductive reagents.

Silicon-Hydrogen bond activation chemistry has been a topic of interest for many years, especially due to the ability of Si-H σ-bond coordination to transition metals[108-112].

The formation of stable M(dσ)–Si-H(σ) complexes have been well studied and characterised and the process of complexation is believed to proceed as illustrated on **figure 5**. Theoretical investigations, IR and other spectroscopic methods have confirmed the theory about the Si-H σ donation as well as the metal(d)-π backbonding. Formation of a σ-complex followed by an oxidative addition step of the Si-H bond is one of the key steps in the mechanism in hydro and/or silylation reactions.

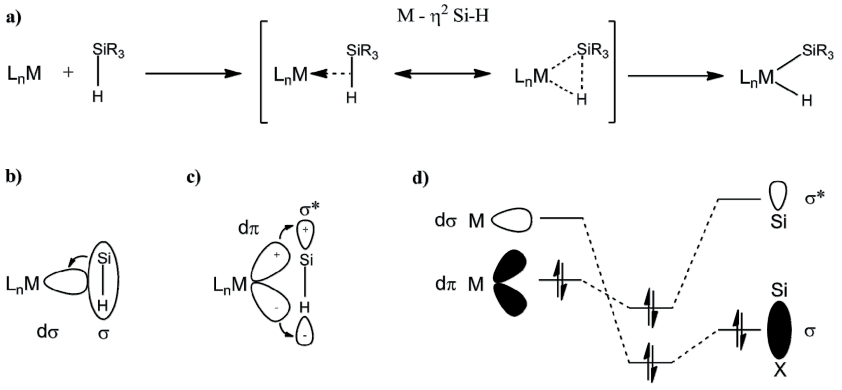
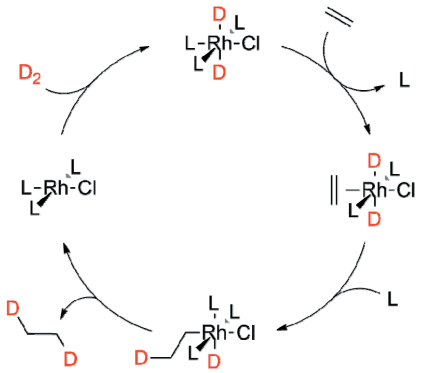


Figure 5. ***a)*** The reaction scheme for the oxidative addition of a silicon-hydrogen bond to a transition metal (M). The transition state involves the initial formation of a M(dσ)←Si-H(σ) complex followed by the oxidative addition. ***b)*** Illutration of the M(dσ)←Si-H(σ) bonding molecular orbitals. ***c)*** Illustration of the M(dπ)→Si(σ*) bonding orbitals. (σ* = anti bonding orbital). ***d)*** The molecular orbital diagram of the bonding orbitals and energy levels.

Olefinic Hydrogenation with Homogeneous Rhodium(I) Complexes

The application of homogenous rhodium catalyst has been reported for hydrogenation and tritiation of olefinic substrates[113, 114]. Rhodium is one of the most important transition metals for this reaction and it works at relatively low hydrogen pressures. The Wilkinson's catalyst ($(Ph_3P)_3RhCl$) was developed in 1966[115] and was shown to hydrogenate a variety of olefinic substrates when dihydrogen was employed.

The mechanism for this hydrogenation is well understood[116] and proceeds through the catalytic cycle illustrated in **scheme 9**. This hydrogenation method has also been utilized in this thesis for hydrogenation of a lipid conjugated peptide. Please refer to the result section for details.



Scheme 9. Mechanism for homogenous olefinic hydrogenation with Wilkinson's catalyst (L = Ph_3P).

The mechanism in short; The 16 electron square planar 'ground state' complex undergoes oxidative addition of dihydrogen to the dihydridorhodium(III) (18 e^-) species. The subsequent dissociation of L and π-complexation of the alkene is then the next step in the hydrogenation process.

One of the key advantages of this catalyst is that it does not affect functional groups such as CHO, OH, CN, Cl, COOH and O, which makes this applicable for use with peptides and proteins. Isotopic scrambling is also observed with this hydrogenation catalyst as illustrated in the result section from the labelling of a conjugate peptide. Also refer to the results presented in part I of this thesis for another examples of isotopic scrambling.

Results

Description and Synthesis of the Peptide Substrates for Hydrogen Labelling

Two substrates/model peptides were synthesised for homogeneous hydrogen labelling experiments. For this standard SPPS was employed, using the Rink amide resin for synthesis of an enkephalinoid peptide and the Wang resin for synthesis of Angiotensin-I. The two substrates are shown on **figure 6**.

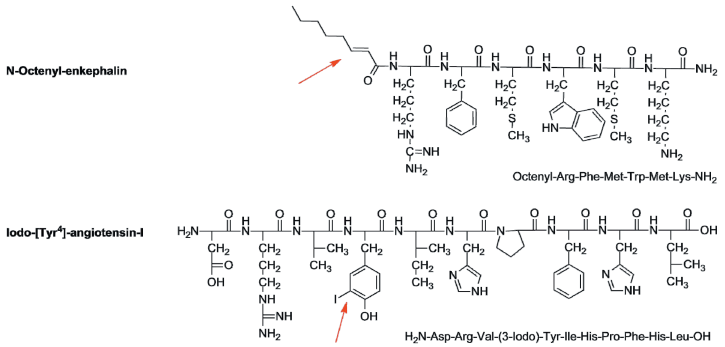


Figure 6. The two synthesised peptide substrates (model compounds) for homogenous hydrogen labelling , N-octenyl-enkephaline and Iodo-[Tyr^4]-angiotensin-I. The red arrows indicating the target of labelling.

N-octenyl-enkephalin

The idea behind the unsaturated lipid conjugated substrate (N-octenyl-enkephalin) was investigation of homogeneous hydrogen labelling of peptides on a conjugate part.

The acetylated hexamer peptide of the sequence Ac-Arg-Phe-Met-Trp-Met-Lys-NH_2 (named acetylines[117] – *acetyl*ated enkepha*lines*) was found to have very high affinity towards opioid receptors. In a large screened chemical library of more than 52 million hexapeptides, this peptide was found to have selective antagonistic properties for the μ, κ_3, δ opioid receptors with the impressive IC_{50} values 1.9 nM, 0.7 nM and 24.7 nM respectively[118]. It bears no resemblance to any known native opioid peptides, but it could be a promising therapeutic peptide. The biology is, though, outside the scope for this work. Here it is used as a model peptide for demonstration of double bond hydrogenation. Also in contrast to the increasing demand in the medicinal industry for tracing conjugate parts of "new" polymer drugs, this peptide could be imagined as a suitable model substrate for tritiation. For convenience this synthetic peptide will be named *N-octenyl-enkephalin* throughout this thesis, although it has no structural similarity to the opioidic peptide enkephalins[119].

Iodo-[Tyr^4]-Angiotensin-I

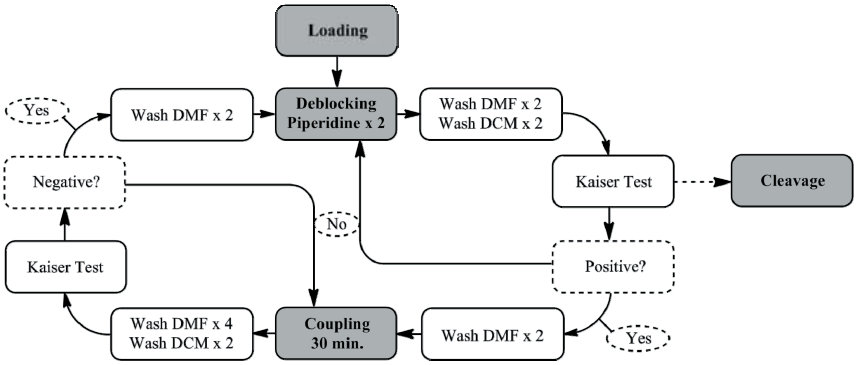
The other substrate selected and synthesised was iodo-[Tyr^4]-angiotensin-I. A 3-iodo-tyrosine residue was incorporated into the backbone of the peptide in the synthesis. This peptide is a ten-mer biological relevant peptide, though without known therapeutic value. It was chosen as a substrate for development of new homogenous hydrodehalogenation reactions. The peptide was also chosen as a model peptide, as it contains a variety of functional groups that could interfere in homogenous catalysis.

General Materials and Methods for SPPS. The peptides were synthesised by manual SPPS using Fmoc protection strategy with HATU/DIPEA as the coupling reagents and the DIC asymmetrical anhydride method for loading of the Wang resin. Manual reaction vessels with glass-filters were used with rotation (shown in **figure 7**). Protected amino acids, resins and reagents were used as received from GL Biochem (Shanghai). The actual loading of the resin was not determined; instead the specifications from the vendor were used for the stoichiometric reagent and theoretic yield calculations. For the Wang resin the loading was specified to 1 mmol/gram resin and for the Rink amide resin 0.5 mmol/gram resin.

Figure 7. Manual SPPS reaction vessel (5 ml) from Peptides International Inc.

General Procedure. Loading of the Wang resin was performed using the symmetrical anhydride loading method employing diisopropylcarbodiimide (DIC) as the reagent. The Rink amide resin was initially Fmoc deblocked and loaded using standard HATU/DIPEA coupling. In each amino acid coupling 4 eq. Fmoc protected amino acid, 3.9 eq. HATU and 8 eq. DIPEA were used. After each coupling and Fmoc deblocking steps a Kaiser test was performed for a qualitative evaluation of the coupling/deblocking quality. In case of blue/purple-ish beads after an amino acid coupling, the coupling was incomplete and consequently a second coupling was performed. The Kaiser tests were positive (blue) after all Fmoc deblockings. For further details about the peptide synthesis (procedure and results from Angiotensin-I synthesis) please refer to the supplemental information of the manuscript in appendix.

The peptide synthesis flow-diagram used in these syntheses is illustrated in **scheme 10**.



Scheme 10. General flow diagram for SPPS of the two substrates.

N-(oct-2-enyl)-enkephaline. In a manual SPPS reaction vessel Fmoc-Rink amide resin (678 mg) with a specified loading of 0.5 mmol/gram were deblocked with 20% piperidine in DMF (4-5 ml). In a small beaker Fmoc amino acids (4 eq.) and HATU (3.9 eq.) were balanced out and dissolved in a minimum amount of dry DMF (typical 4-5 ml) then DIPEA added. This acylation reagent mixture was used in the sequential amino acid couplings (30-40 min.) and piperidine (2 x 4-5 ml 20% piperidine in DMF) in the Fmoc deblockings. The acylations were performed in following order; FmocNH-Lys(Boc)-OH, FmocNH-Met-OH, FmocNH-Trp(Boc)-OH, FmocNH-Met-OH, Fmoc-Phe-OH, Fmoc-Arg(Pbf)-OH and at the end 2-octenoic acid. After completion, the peptide was cleaved with reagent B, (88 % TFA, 5% H_2O, 5% phenol and 2% triisopropylsilane). After removal of the reagents under reduced pressure, resolvation in a minimum amount of 40% MeCN with 0.1% TFA and filtration of insoluble compounds through a Wattman GFC filter; the crude material was subjected to preparative HPLC for purification.

Preparative RP-HPLC was performed on a Knauer Smartline. (Components: Manager 5000, Pump 1000, UV Detector 2500) with a Eurosphere C-18 column (20 x 250 mm) at ambient temperature (20^{o}C). Mobile phase: A (5% MeCN, 0.1% TFA) and B (99.9% MeCN, 0.1% TFA). Gradient: 0-4 min (0%B), 4-17 min. (0%B to 35%B), 17-25 min. (35%B to 100%B), 25-28 min. (100%B), 28-29 min. (100%B to 0%B), 29-33 min. (0%B). Flow: 10 ml/min.

The peptide was collected at 27.6-28.3 min. and the combined fractions lyophilized, yielding 18 mg (theoretical 5% of specified loading of the resin). The peptide was analysed to have a purity >95% (**figure 8**).

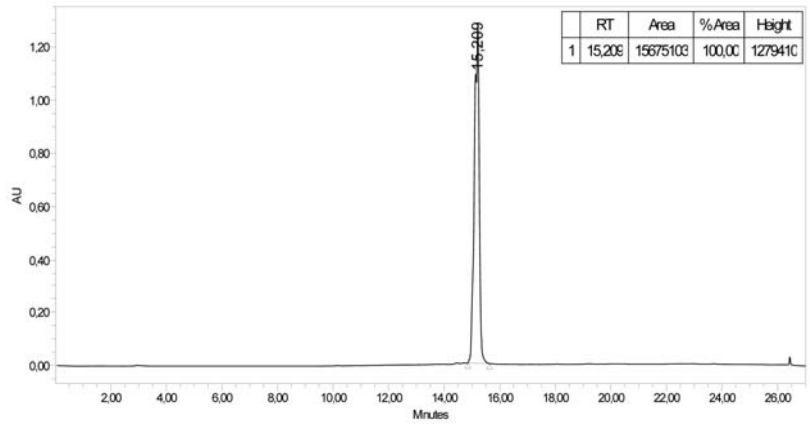


Figure 8. Analytical HPLC of N-(oct-2-enyl)-enkephaline after purification.

Analytical HPLC was performed on a Waters Breeze system. Pump: Waters 1525, Binary Pump. UV Detector**:** Waters 2787, Dual λ Absorbance Detector with the Phenomenex Luna C-18 column at ambient temperature (20^{o}C). Mobile phase: A (5% MeCN, 0.1% TFA) and B (99.9% MeCN, 0.1% TFA). Gradient: 0-3 min (0%B), 3-15 min. (0%B to 60%B), 15-20 min. (60%B to 100%B), 20-23 min. (100%B), 23-24 min. (100%B to 0%B), 24-27 min. (0%B). Flow: 1 ml/min.

Iodo-[Tyr^{4}]-Angiotensin-I and Angiotensin-I. Experimental details about synthesis, materials and methods are described in details in the Supplemental Information, in Appendix I.

Olefinic Hydrogenation of N-Octenyl-Enkephalin

Homogenous hydrogenation of unsaturated compounds is an important tool in organic synthesis and it is also well understood[120]. In this section, the results from the homogenous hydrogen labelling of the conjugated *N*-octenyl-enkephalin are described in detail.

As mentioned in the beginning of Part II of this thesis, hydrogenation of peptides and proteins in aqueous solvents would be desirable. For the investigation of hydrogenation in water a commercial water soluble rhodium(I) hydrogenation catalyst were employed in these experiments and compared to the original Wilkinson catalyst (see **figure 9**).

The experiments were designed as qualitative investigations of hydrogen incorporation into the peptide conjugate part.

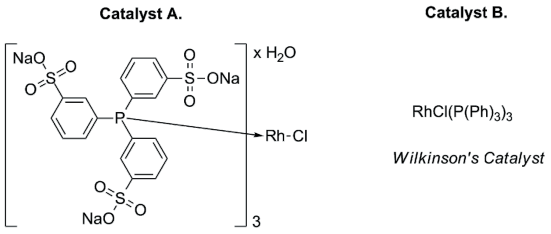
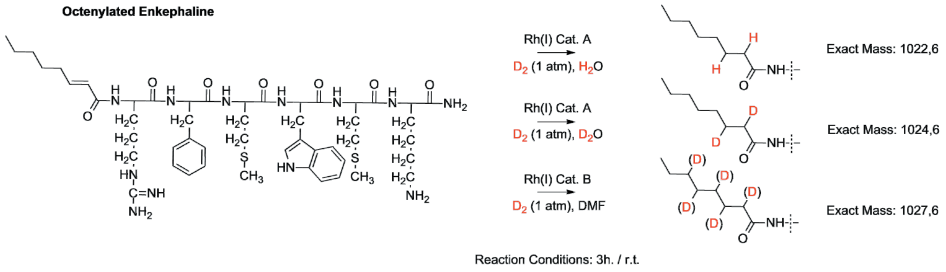


Figure 9. Catalyst used for homogenous deuterium labelling of 2-octenylated enkephaline. Catalyst A: RhCl(*m*TPPTS)$_3$, where *m*TPPTS = *tris*(*meta*-sulfonatophenyl)phosphine. Catalyst B: RhCl(PPh$_3$)$_3$ – *tris*(triphenylphosphine)rhodiumchloride.

The overall results from the hydrogenation reactions are listed in **scheme 11** and the incorporation of proton- and deuterium- atoms into the substrate were measured by ESI-MS as shown on **figure 10** (next page).



Scheme 11. Results from the hydrogenation reactions. The positions of incorporated hydrogen are proposed to be in the conjugated octenoic acid functionality of the octenylated enkephaline.

In the reactions using aqueous solvents the hydrogenated product corresponds to addition of two protons when H_2O was used, and two deuterons when D_2O were

used, as the solvent. In the non-aqueous reaction where the Wilkinson's catalyst was employed in DMF as the solvent, multiple deuterium incorporations (1-6 D) was observed.

In these reactions the hydrogen donor in the aqueous hydrogenation were shown to originate from the protic solvents when the water soluble rhodium(I) catalyst ((*m*TPPTS)$_3$RhCl) were employed.

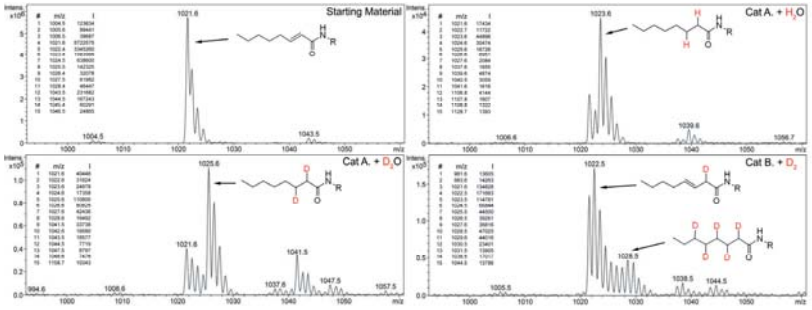
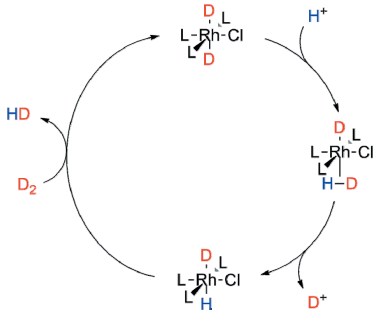


Figure 10. Mass spectra of the products from the hydrogenation reactions after 3 hours at room temperature.

The use of the water soluble hydrogenation catalysts of both rhodium and iridium and the isotope source from protic solvents has also recently been reported in the literature[121]. The general accepted mechanism for the H/D exchange of the rhodium catalyst is illustrated on **scheme 11**. An oxidative addition of deuterium (D_2) followed by an X^+/D exchange is the reported explanation for these observations (where $X = H^+$ or D^+).

Interestingly this means that the hydrogen donor could be aprotic solvent and the electrons for the oxidation be dihydrogen / dideuterium. The mechanism for the H/D exchange on the rhodium(III) catalyst is proposed to proceed as shown on **scheme 11** as it was presented in the literature[122].



Scheme 11. Illustration of proposed catalytic H/D exchange cycle of the dideuterio-rhodium(III) complex[122].

It must be concluded that the water soluble rhodium catalysts are not feasible as aqueous tritiation catalysts. The application of homogenous (rhodium/iridium) catalysts for hydrogen labelling of olefinic substrates is a useful method for deuteration and tritiation reactions in non-protic solvents. It has been illustrated that the employment of Wilkinson's catalyst in DMF is a promising hydrogenation catalyst for tritium labelling of conjugated peptide fragments containing unsaturated carbon-carbon bonds. Indications from the results here suggest isotopic scrambling and multiple deuterium incorporations. The isotopic scrambling mechanism is proposed in the result section of part I and similar mechanism can be imagined with the Wilkinson's catalyst in this hydrogenation.

General Hydrogenation Procedure. In a round bottomed flask (1 ml) the substrate *N*-octenyl-enkephalin (1-2 mg) and catalyst (3-4 mg) were dissolved in the solvent (0.5 ml). The flask was mounted onto the manifold system and the reaction mixture cooled to -192^{o}C with liquid nitrogen. The reaction was evacuated to below $5*10^{-6}$ bar and flushed with argon twice. The mixture was warmed to room temperature and stirred for 5 min. (for deoxygenation) before it was recooled with liquid nitrogen.

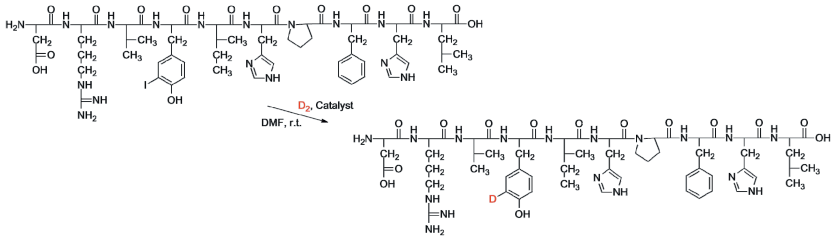
The flask was evacuated to below $5*10^{-6}$ bar and deuterium gas (D_2) was introduced onto the manifold system and over the reaction mixture. The reaction was allowed to warm upon room temperature and the deuterium pressure in the reaction was adjusted to approximately 1 atm. (~ 1050 mbar).

The reaction mixture was stirred for 3 hours at room temperature (20^{o}C) and the reaction stopped. Semi-purification was performed before MS analysis. Labile deuterium were removed by 2 sequential lyophilisations with H_2O:MeOH (1:1, 5 mL). The lyophilised products were solubilised in H_2O (5 mL) and a few drops of MeCN added then applied onto a preactivated C-18 plus SEP-PAK®. The SEP-PAK was washed with H_2O (5 ml) and the products eluted with MeCN:H_2O:TFA (95:5:0.1, 5 ml). After lyophillation and dissolution of the peptide product in H_2O the hydrogen incorporation was determined by ESI-MS.

Gaseous Deuteriodeiodination of Iodo-Angiotensin-I

One of the preferred tritium labelling methods for small molecules and peptides is the hydrodehalogenations reaction using a heterogeneous catalyst on a solid support and tritium gas (T_2).

When this method is employed for labelling of peptides it could be imagined that the choice of catalyst and/or support is important for the yield. The yields will dramatically decrease when this method is used on larger substrates (proteins). As described earlier, it is speculated to be caused by clotting/sticking of the substrate to the catalyst or support[2] or by inaccessibility to the active site (Ar-I). Initially, a series of different catalyst and supports were investigated with deuterium gas (for deuteriodeiodination of iodo-angiotensin) to get a perspective of these effects. In the same series, a few homogenous catalysts were also applied for comparison. The overall reaction is depicted in **scheme 12** and the results summarised in **table 1**.



Scheme 12. Heterogeneous deuteriodeiodination of iodo-[Tyr[4]]-angiotensin-I.

Catalyst	Solvent	D_2 [mbar]	Reaction Time	Yield	Recovery	Specific Labelling
			Heterogenous Catalysts			
5% $Pd/BaSO_4$	DMF	750	2 hours	51%	31%	100%
5% $Pd/BaSO_4$	DMF	1050	2 hours	70%	25%	100%
5% $Pd/BaSO_4$	DMF	1230	2 hours	58%	0%	100%
10% Pd/C	DMF	1000	2 hours	63%	0%	100%
5% $Pd(OH)_2/C$	DMF	1230	2 hours	48%	0%	100%
10% Pt/C	DMF	800	3 hours	Trace	-	-
5% Ru/C	DMF	800	3 hours	0%	-	-
			Homogenous Catalysts			
$PdCl_2$*	DMF	1050	2 hours	82%	19%	100%
$Pd(OAc)_2$*	DMF	1230	2 hours	79%	0%	100%
$Pd(PPh_3)_4$	DMF	750	2 hours	0%	0%	-
$RhCl(PPh_3)_3$	DMF	800	72 hours	0%	0%	-

Table 1. Selected entries from parallel heterogeneous deuteriodeiodination of iodinated angiotensin-I. * Palladium(II)chloride and palladium(II)acetate are both initially homogenous but in the reactions with hydrogen gas (D_2) the reaction turns almost immediately black and becomes heterogeneous.

The table of results show the yield and the recovery yield. These corresponds to the isolated deuterio- and iodo- peptides amounts respectively. Yields are calculated from the intensities in ESI-MS, assuming identical ionization efficiency of each peptide in the electrospray interface.

The results from these deuteriodeiodination reactions give a somewhat clear image of the performance of the different catalyst and supports. In summary, as expected, the heterogeneous palladium catalyst is most effective in this hydrogenation reaction. When the palladium(II) catalysts (palladium chloride and palladium acetate) were employed, the reaction remained clear orange and homogeneous until the reactions were subjected to hydrogen gas. The palladium(II) was reduced and almost instantly turned black and inhomogeneous. For comparison the phosphine ligated Pd(0) catalyst, *tetrakis*(triphenylphosphine)palladium(0), was tested in same D_2 reaction. No deuteration was observed with the use of this catalyst and the similar result with Wilkinson's catalyst. These latter reactions though stayed homogeneous.

General procedure for gaseous deuteriodeiodination of iodo-angiotensin-I.

In a round bottomed flask (1 ml) the substrate (2-4 mg, iodo-angiotensin-I) was dissolved in DMF (0.5 ml) and a small amount of catalyst (2-4 mg) added. The flask was mounted onto the manifold system and frozen with liquid nitrogen. It was then evacuated to below $5*10^{-6}$ bar, flushed twice with argon and allowed to warm to room temperature while stirring for 5 minutes (for degassing of solvent). Subsequently the reaction mixture was frozen with liquid nitrogen, evacuated to below $5*10^{-6}$ bar, before deuterium gas was introduced over the reaction. After heating to room temperature (20^{o}C) the hydrogen pressure was noted and the reaction left for stirring for an appropriate time. The products were then analysed after a SEP-PAK semi-purification for the removal of both DMF and catalysts. The distribution of product and starting material was measured by ESI-MS, the yield and recovery calculated by weight and specific labelling determined by ESI-MS.

Silane and Palladium(0) Hydrodeiodinations of Iodo-Angiotensin-I.

Moving towards the use of homogeneous catalysts for hydrodeiodination of the iodinated peptide substrate (iodo-angiotensin-I) - a suitable hydrogen donor was needed. As it was shown by the gaseous D_2 experiments (in the previous section), none of the homogeneous catalysts were able to hydrogenate to give the deuterated products.

A hydridic hydrogen donor, on the other hand, could be believed to perform the H/I exchange in presence of a suitable cross coupling catalyst. One requirement for the choice of hydride was that it could be synthesised from hydrogen gas and eventually be transferred to tritium (T_2) chemistry. The metalloids boron, silicon and tin allow for mild coupling with diverse electrofiles in presence of sensitive functionalities of various kinds (which is needed for peptides). Also, because boron, silicon and tin tritides can be synthesised from lithium tritide (see Part I) these were attractive to investigate with homogeneous catalysts. Interestingly, the preparation of silicon tritides and their applications as reducing agents in a palladium(0)-aryl triflate coupling were reported. As briefly described previously, silanes are suitable tritium labelling reagents in a palladium-aryl triflate coupling reaction[123].

We therefore set out to investigate silicon hydrides as potential reductive hydrodeiodination and hydrogen labelling reagents. Two silanes, the electron rich triethylsilane (Et_3SiH) and the electron deficient triethoxysilane (EtO_3SiH), where acquired from commercial vendors and employed with the substrate with and without a palladium catalysts.

The results from these initial/preliminary silane and palladium(0) hydrodeiodination reactions are listed in **table 2**.

Catalyst	Solvent	Silane	Amount	Reaction Time	Specific Labelling	Conversion
Pd(0) mediated hydrodehalogenation with Silane						
None	DMF	Et_3SiH	50 uL	24 hours	-	0%
None	DMF	EtO_3SiH	50 uL	24 hours	-	0%
$Pd(PPh_3)_4$	DMF	Et_3SiH	50 uL	24 hours	100%	14%
$Pd(PPh_3)_4$	DMF	Et_3SiH	50 uL	96 hours	62%	100%[A]
$Pd(PPh_3)_4$	NMP	Et_3SiH	50 uL	24 hours	100%	77%
$Pd(PPh_3)_4$	DMF	EtO_3SiH	100 uL	2 hours	45%	100%[A]
$Pd(PPh_3)_4$	NMP	EtO_3SiH	100 uL	24 hours	70%	100%[A]
$Pd(PPh_3)_4$	NMM	EtO_3SiH	100 uL	24 hours	-	0%
$Pd(PPh_3)_4$	PEG_{400}	EtO_3SiH	50 uL	24 hours	-	5%[B]
$RhCl(PPh_3)_3$	DMF	EtO_3SiH	100 uL	2 hours	-	0%

Table 2. Results from the protiodeiodination reaction using homogeneous palladium and commercial silanes - initial screening of reactants. Reactions were carried out in a total reaction volume of 1 ml and stirred at room temperature for the indicated time (20°C).
[A] All starting material was consumed, but additional unidentified products observed in MS.
[B] Aproximate conversion, peptide visual insoluble in PEG400 and PEG observed in MS.

From the result table the hydrodeiodination reactions were successful when silanes were combined with a palladium(0) catalyst and no product was observed without palladium(0). It must be noted that reactions were observed to be homogeneous in all cases.

For optimisation of reaction conditions a few parameters were changed, such as the solvent, reaction times and reagent. The solvents that gave the best conversion were *N*-methylpyrolidone (NMP), dimethylformamide (DMF), but for the other PEG_{400} and *N*-methylmorpholine (NMM), no or little conversion was observed. Long reaction times with triethylsilane and the use of triethoxysilane also resulted in additional/unidentified products. As an example the mass spectrum from the reaction with $(EtO)_3Si^1H$ in NMP is illustrated on **figure 11**.

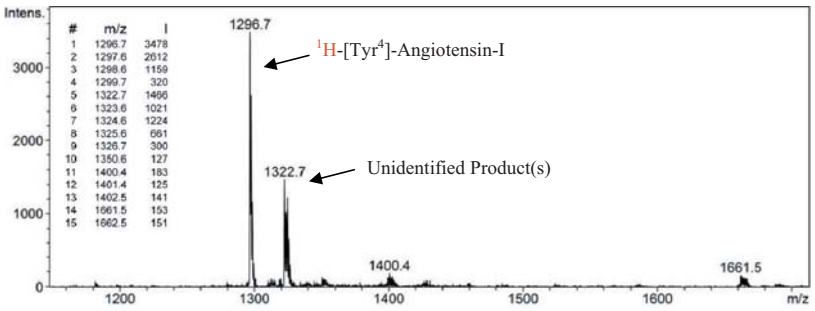


Figure 11. The mass spectrum from the hydrodeiodination of iodo-[tyr⁴]-angiotensin-I, with large excess of $(EtO)_3Si^1H$ and mediated by tetrakis(triphenylphosphine) palladium(0).

Part II. Development of New Tritium Labelling Methods for Peptides.

General Procedure for ^{1}H-Silane Palladium(0) Hydrodeiodination.

Handling and mixing of reagents were performed in a custom made argon filled glove box. The substrate iodo-[Tyr^{4}]-angiotensin-I (2-4 mg, 1-3 µmol) and the catalysts Pd(0) (or Rh(I)) (2-4 mg, 2-4 µmol) was added to vials (10 ml size), then capped and weighed outside the glove box. The substrate was dissolved in DMF (0.5 ml), and all solution transferred to the vial containing the catalyst. After gentle agitation of the reaction mixture for a couple of minutes, for total dissolution the catalyst, the silane (50 – 100 µl, 300-600 µmol) was added. Subsequently the reaction mixture was transferred to a round bottomed flask loaded with at stirbar and mounted onto the manifold system. It was then cooled to -192^{o}C with liquid nitrogen, evacuated to below $5*10^{-6}$ bar and the flask filled with argon (to a pressure slightly below 1 atm.). The products were then analysed after a SEP-PAK semi-purification for the removal of DMF (total removal of the homogeneous catalysts were not possible by SEP-PAK). The products formed and starting material in the reaction was analysed by ESI-MS. Conversion was calculated by product ion-counts and dividing with the total added ion-counts on ESI-MS, expecting the same ionization efficiency of the products.

Conclusion

The use of hydridosilanes in combination with *tetrakis*(triphenylphosphine)-palladium(0) turned out to be successful in these initial hydrodeiodination reactions. As presented here, a very large excess of silane is used with respect to the substrate and with these amounts this method cannot be transferred directly to tritium (T_2) chemistry. The amount of synthesised tritiosilanes needed to be kept in the range of 100-200 micromoles for safety reasons. This chemical amount would correspond to a theoretical 2.9-5.8 Ci of radioactivity (SA 28.9 Ci/matom T). Also tritiated butane would be formed as a by-product in the lithium tritide (LiT) synthesis (see Part I for details). For convenience, in the light of the radioactivity amounts, this chemistry was first attempted with small scale deuterium (D_2) gas chemistry as a model for tritium. The general approach for the synthesis of deuteriosilane is illustrated in **scheme 13** (for experimental details about the synthesis and analysis of deuteriosilanes, please refer to appendix I) and the synthesis of LiD on **figure 12**.

Scheme 13. General synthesis of triethyldeuteriosilane.

The pictures on **figure 12** are showing the reaction vessels (3 ml) in the three steps of lithium deuteride synthesis.

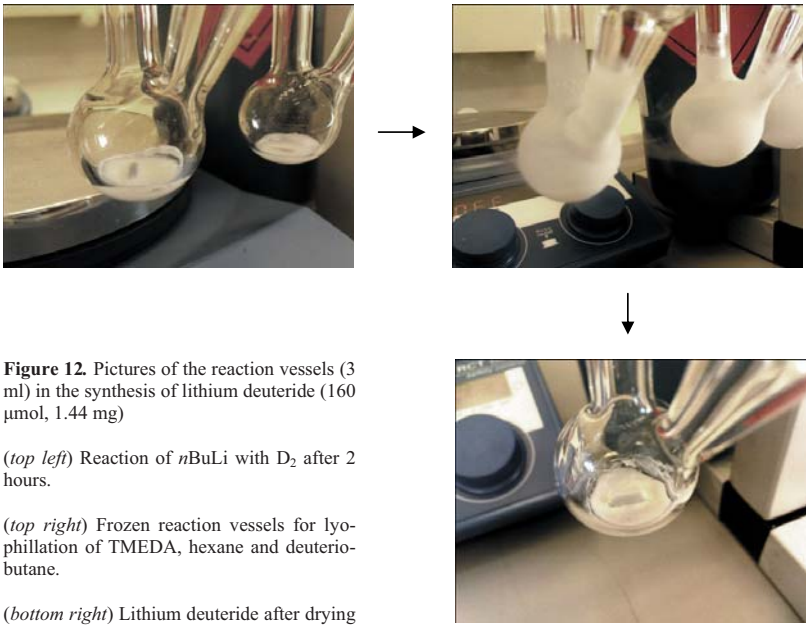


Figure 12. Pictures of the reaction vessels (3 ml) in the synthesis of lithium deuteride (160 μmol, 1.44 mg)

(*top left*) Reaction of *n*BuLi with D_2 after 2 hours.

(*top right*) Frozen reaction vessels for lyophillation of TMEDA, hexane and deuteriobutane.

(*bottom right*) Lithium deuteride after drying under vacuum.

When the silane chemistry was transferred to deuterium chemistry the conversion of the iodinated peptide dramatically decreased with the use of the *tetrakis*-(triphenylphosphine)palladium(0) catalyst. This was why, we initiated a screening of a series of phosphine ligands in combination with a palladium(II) source.

The tuning of the phosphine ligands and the reaction conditions eventually gave full conversion of iodo-angiotensin to the corresponding deuterated peptide. These optimization studies as well as the results have been described in the manuscript in appendix I.

In short, we found that with the use of the bulky electron rich tri(*tert.*-butyl)phosphine as the ligand and subsequent addition of fluoride (for cleavage of hydroxyl-silylation products) successful hydrogen labelling occurred.

Silane and Palladium(0) Deuteriodeiodination of Glucagon-Like-Peptide-1

The native unconjugated GLP-1 peptide was chosen as a suitable and larger substrate for the palladium(0) deuterio-silane labelling reaction. This was done in collaboration with our partners at Novo Nordisk A/S (NN).

Glucagon-Like-Peptide-1 is a natural (pancreatic secreted and hormonal) peptide involved in regulation of glucagon plasma levels. This 30-mer peptide (residue 7 - 37) has thus gained increasingly interest as a potent therapeutic target for the treatment of diabetes mellitus, and the number of publications about this peptide has grow exponentially over the last decade[124]. The conjugated GLP-1 peptide drug, Liraglutide®, marketed by Novo Nordisk A/S, was recently (January 25th 2010) approved by FDA as a therapeutic drug for regulation of blood sugar levels[125]. This conjugated peptide drug is the second GLP-1 analog to be adopted onto the American market.

A crystal structure of GLP-1 complexed with the GLP-1 receptor was also recently published[126]. The structure is here illustrated as the red alpha helix in **figure 13**. The blue structure represents the GLP-1 G-coupled trans-membrane receptor.

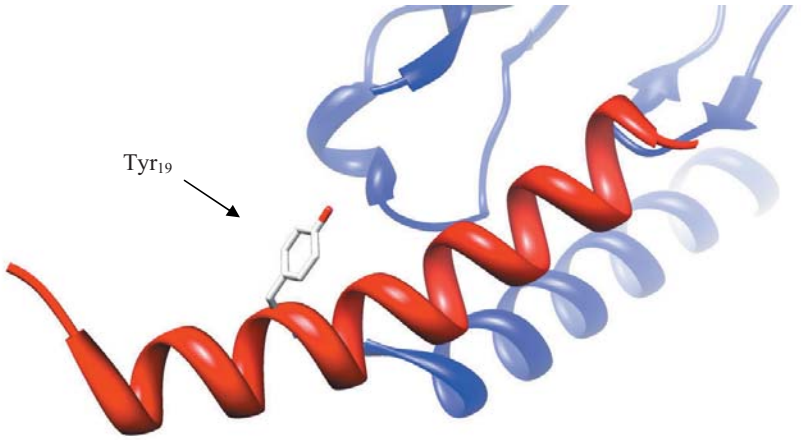


Figure 13. An illustration of the crystal structure of GLP-1 in complex with the extracellular domain of the GLP-1 receptor[126]. The red helical structure is GLP-1 where the tyrosine residue, highlighted in the figure, was the target for deuterium and tritium labelling. The illustration was reproduced from the PDB file (3IOL.pdb) and image created with the UCSF Chimera software[94].

Tritium labelling of GLP-1 and the conjugate drug – Liraglutide® was an essential tool for ADME studies and for acquiring of the marketing license. The methods for labelling Liraglutide® on both the conjugate and peptide part by gaseous heterogeneous olefinic tritiation and tritiodeiodinations respectively, have been published[37].

As mentioned earlier both parts of these "new" type polypeptide drugs have to be accounted for in distribution, metabolic and excretion studies in the preclinical trials. The structure of Liraglutide and the structural targets for tritiation, at the isotope laboratories of Novo Nordisk (NN), are both illustrated in **figure 14** (next page).

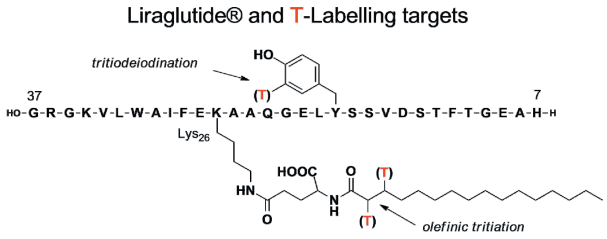
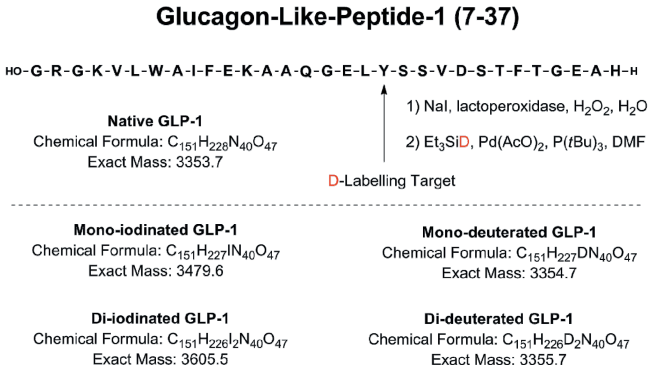


Figure 14. The amino acid sequence and the conjugate part of the peptide drug, Liraglutide®, are here presented. The targets for tritiation are illustrated with the arrows and the method of tritiation in caption.

The tritium labelled GLP-1 is routinely prepared at the isotope laboratories at NN, so the iodinated GLP-1 precursor was readily available. At the isotope laboratories at NN we prepared this precursor by an enzymatic iodination with the use of sodium iodide, hydrogen peroxide and lactoperoxidase. This is a proven method for iodination of proteins and other macromolecules in aqueous solutions[127]. The experimental details are, for confidentiality reasons, here not described in details. It can be mentioned, though, that a standard tritiation was performed for, educational and demonstration purposes.

Results. The general approach for deuterium labelling of GLP-1 is shown below in **figure 15**. For elucidation of the formed products, the exact masses of the substrate and product peptides are listed, for the MALDI-TOF analyses. The experimental procedure for this labelling was identical to the general procedure for deuterium labelling of iodinated angiotensin. This procedure is described in the appendix.



Scheme 15. The procedure for deuteriodeiodination of GLP-1 and the calculated exact masses for the relevant peptide products for the MALDI-TOF analyses.

After reaction the peptides were analysed on a mass-spectrometer. MALDI-TOF-MS was recorded on a Bruker Reflex IV MALDI-TOF Spectrometer using 2,5-dihydroxybenzoic acid (DHB) in MeOH as matrix. The results are illustrated on the following spectra in **figure 16** MS of the native GLP-1 and in **figure 17** MS from the reaction.

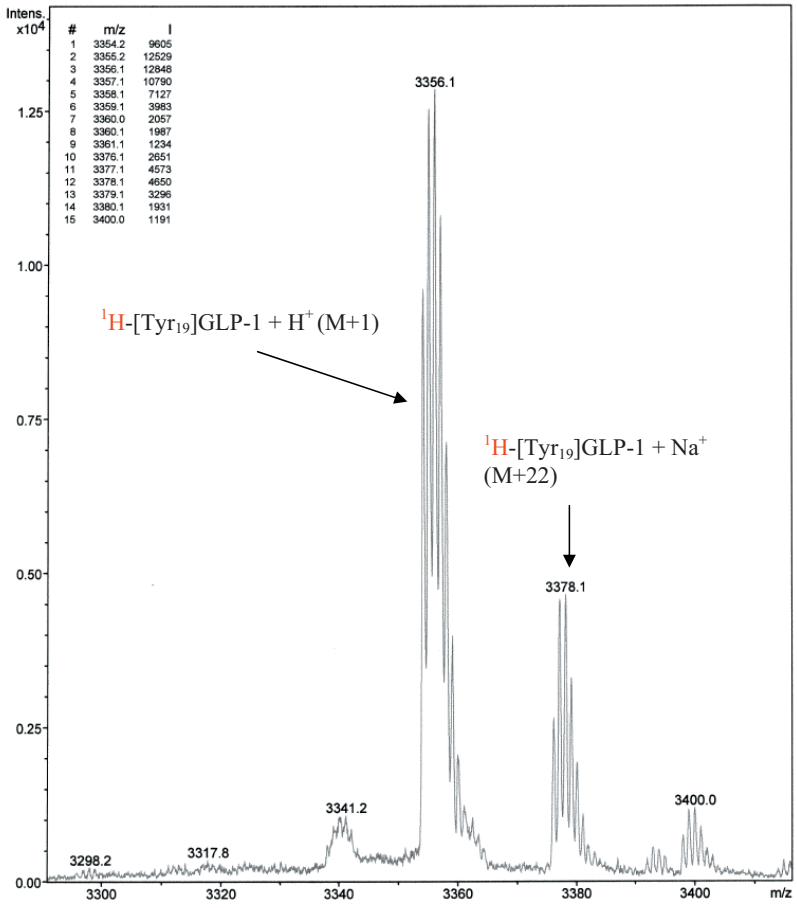


Figure 16. Mass spectrum of Native GLP-1 acquired on MALDI-TOF

The mass spectrum shown in **figure 16** illustrates the m/z of the monoprotonated ($M + H^+$) and the sodium adduct ($M + Na^+$) native GLP-1 peptide. This spectrum was used as the standard for the deuterium labelling.

After the enzymatic iodination of GLP-1 a distribution of native, mono- and di-iodinated GLP-1 analogs were present in a mixture. A purification using preparative

HPLC was performed for a pure mono-iodinated peptide substrate in order to label with tritium. (experimental details for purification is not presented here).

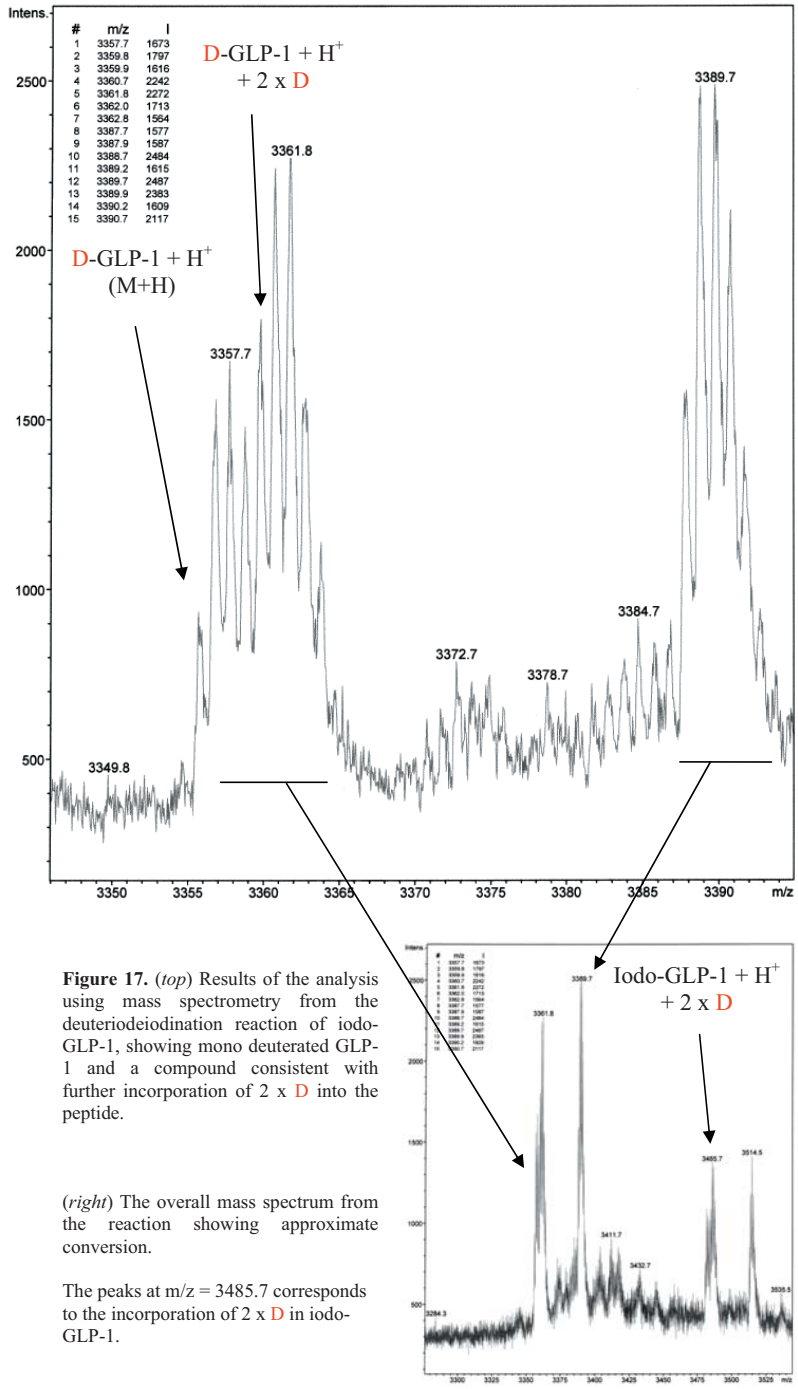


Figure 17. (*top*) Results of the analysis using mass spectrometry from the deuteriodeiodination reaction of iodo-GLP-1, showing mono deuterated GLP-1 and a compound consistent with further incorporation of 2 x D into the peptide.

(*right*) The overall mass spectrum from the reaction showing approximate conversion.

The peaks at m/z = 3485.7 corresponds to the incorporation of 2 x D in iodo-GLP-1.

Conclusion from the GLP-1 deuteration

The objective for labelling of iodinated-GLP-1 was selective deuteriodeiodination with the palladium(0) deuterio-silane method which is presented in appendix.

The results from these reactions were not absolutely conclusive. This was due to observations based on the analysis of reaction products from the deuteration of GLP-1. Some of the compounds, detected by MALDI-TOF, correspond to multiple deuterium incorporations into the molecule.

Possible incorporation of 2 deuterium atoms into the iodinated substrate was also observed. A plausible theory for this observation could be hydrogenation of an unsaturated moiety in the peptide backbone; this would essentially give a mass of the product of +4 m/z units (it would be speculative to suggest which amino acid). For further investigation of this observation experiments must be conducted on selected aminoacid residues not present in the angiotensin-I substrate *e.g.* tryptophan.

It has not been possible to elucidate the possible products from these labelling reactions and the HPLC analysis of GLP-1 product was all unsuccessful, probably because of palladium adducts or peptide fibril formation.

From the results it has to be concluded that results, from the homogeneous deuterio-silane labelling method of GLP-1, was unsuccessful, since the mono-deuterated GLP-1 was not selectively formed in the reaction.

Silane and Palladium(0) Tritiodeiodination of Iodo-Angiotensin-I

The ultimate goal in development of the homogeneous deuteriodeiodination reactions was transfer of the developed deuteration methods to tritium chemistry. After the setup of a tritium manifold and dismantling of the deuterium gas, several attempts utilizing the silane chemistry with tritium have been attempted.

The optimised reaction conditions for deuterium labelling of iodo-Angiotensin-I were used in the attempt to tritium label of this substrate. For radiochemical safety reasons, synthesis of triethyltritiosilane could not be followed by analytical methods.

Short note about the safety issues

Transferring the deuterium silane to tritium chemistry makes the practical procedures in the laboratory much more laborious. Safety issues - regarding the large amounts of radioactivity used in this chemisty - have a great impact on the chemical approach. One requirement for safe-handling of Ci amounts of tritium and synthesis of tritides is surveillance of tritium contamination levels on surfaces of the laboratory. For safety reasons, tritium contamination of surfaces and content in urine samples must be checked. In short, the work with tritium chemistry must comply with general guidelines and law requirements from the authorities[128].

General Procedure for the Tritium Experiments

Lithium tritide (LiT). A round bottomed reaction vessel (1 mL) with a sidearm, septum and stirbar was mounted on the tritium manifold, evacuated and flame dried. The vessel was flushed with argon gas and evacuated 3 times to ensure an anhydrous and oxygen free vessel. After evacuation to a pressure below $5*10^{-3}$ mbar the flask was filled with carrier free tritium gas (600-700 mbar). TMEDA (50 μL) then *n*BuLi (100 μL, 160 μmol, 1.6M in hexanes) were added to the flask with Hamilton syringes through the septum and almost immediately (within 5 - 10 minutes) a white preticipate of LiT was formed inside the reaction vessel. The reaction was allowed react for additional 2 hours at room temperature until full consumption of tritium gas.

The reaction vessel was cooled with liquid nitrogen and the residual tritium gas absorbed on the uranium waste bed. The tritiated butane (by-product), solvent (hexane) and TMEDA were then lyophillized to a vessel mounted onto the distillation branch.

The formed LiT was further dried under vacuum for complete removal of residual TMEDA yielding offwhite solid LiT (theoretical amount of 160 μmole, 1.44 mg, 4.61 Ci).

The reaction vessel was flushed 3 times with argon to ensure an inert atmosphere and left under an argon atmosphere (800 mbar) before the next reaction.

Triethyltritiosilane (Et_3SiT). A solution of triethylchlorosilane (320 µL, 0.5M in THF, 160 µmole) was added to the prepared lithium tritide. The reaction was left with stirring for 1 hour at room temperature to ensure full consumption of lithium tritide before addition of the peptide and catalyst.

Preparation of the triethylchlorosilane solution (0.5M in THF). Triethylchlorosilane (170 µL, δ = 0.898, 152 mg, 1 mmol) was added to an oven dried vial and diluted with dry THF (1830 µL). This reagent solution was subjected to GC to ensure that there had been no decomposition of the chlorosilane before adding to the prepared lithium tritide.

Tritiodeiododination performed by following procedure. In a homemade argon filled glovebox, iodo-Angiotensin-I (2-3 mg, 1-2 µmol) was solubilised in DMF (0.7 mL) in a vial. To this vial was then added the catalyst* (20 µmol, 100 µL) prepared in toluene. The peptide and catalyst were gently agitated for 5 minutes before adding to the prepared triethyltritiosilane mounted on the tritium manifold. Reaction was left stirring for 3 hours before the reaction was treated with TBAF (160 µmol in dry THF). The reaction was left for another hour then workup of the reaction was performed.

Prior to workup a Sep-Pak® Plus (320 mg) C18 cartridge equipped with a 1.2 µm Whatman GF-C filter was preconditioned with ethanol (5 ml) followed by a flush of distilled water (5 ml).

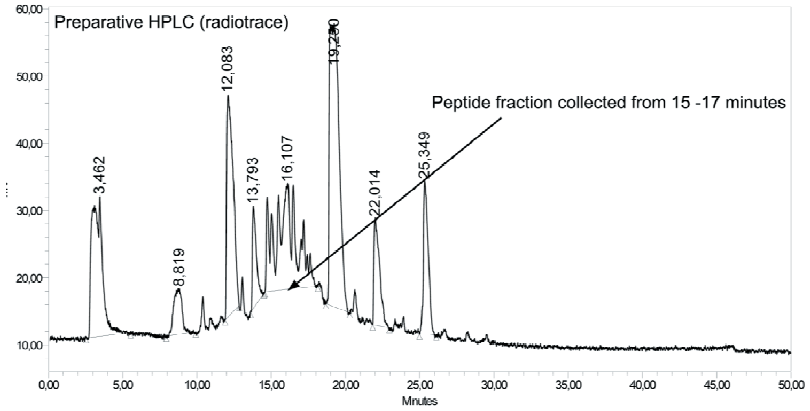
The reaction mixture was then syringed out of the reaction vessel and added to a capped vial containing distilled water (5 ml). This diluted mixture was then filtered through the Whatman filter and applied onto the Sep-Pak®. The filter and Sep-Pak® was then washed with ultra-purified water (5 ml) for the removal of salts and DMF solvent. Afterwards the filter was disposed and the products were eluted using a mixture of 80% MeCN, 20% Water and 0.1% TFA (3 ml). For removal of labile tritium, the products were lyophilized 3 times with 50% MeOH in H_2O (3 ml). The products were then subjected to HPLC for purification and analysis.

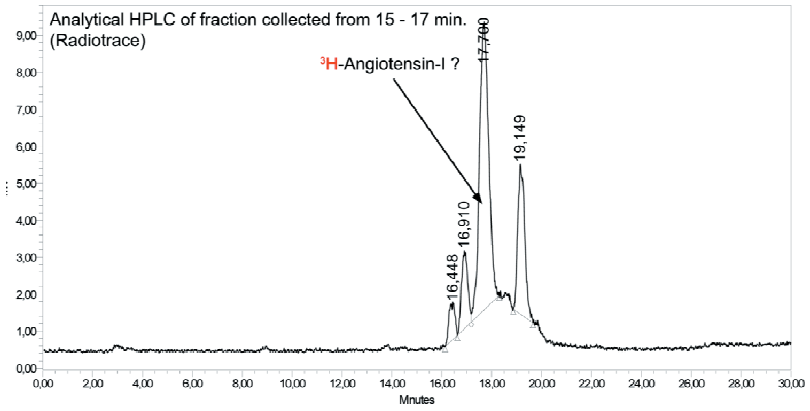
***Preparation of paladium catalyst (Pd(0) + P(*t*Bu)$_3$).** The catalyst was prepared in a vial externally prior to use in a small vial. Palladium(II) acetate was added 5 eq. of tri-*tert.*-butylphosphine (1M in toluene). Of this catalyst solution 20 µmoles was added to the substrate vial and it was left for 10 minutes before transferring to the reaction vessel containing the deuterosilane solution, leaving a pale yellow homogenous reaction mixture. After 3 hours of reaction time 1 eq. of tetrabutylammonium fluoride TBAF (160 µmole, fluoride) was added to the reaction. 30 minutes after this addition the workup of product was performed.

Discussion of Results

From the 5 experimental tritiations performed the results from the last reaction provided the most promising results. As a result of problems with the Triatler HPLC radiodetector and other issues, which will not be discussed here, the first reactions were generally inconclusive. The results presented here are representative of the other performed reactions.

The preparative and analytical HPLC traces from the reaction performed are illustrated in **figure 18**. From the radiotrace of the preparative HPLC appearance of many radioactive substances are observed. Due to the large excess of tritium (160 μmole) in contrast to the amount of substrate (1-2 μmole) other radioactive compounds are also expected seen in the purification. The Sep-Pak semipurification does though remove a large quantity of the radioactive by products, silicates, tritiated solvents and so on. From preliminary HPLC method developments the angiotensin peptides eluted at 15-17 minutes with the preparative methods. Therefore this area of the chromatogram is of particular interest, though fractions of all parts of the chromatogram were collected and measured by liquid scintillation counting for radioactivity quantification.





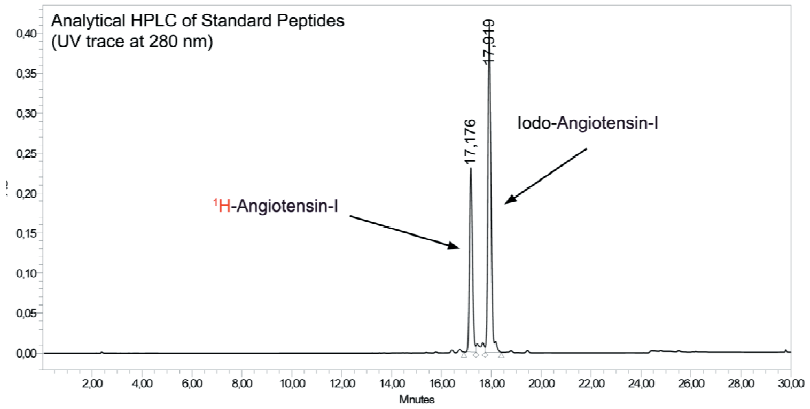


Figure 18. (*top*) Results from preparative HPLC purification, radiotrace the. (*middle*) Analytical HPLC trace. (*bottom*) Analytical HPLC UV trace of Angiotensin-I and iodo-Angiotensin-I.

With LSC the amount of radioactivity in the 15-17 minute fraction was determined to be 64 MBq of which the main peak (17.7 min) in the analytical HPLC trace, could represent the tritium labelled Angiotensin. The delay between the UV detector and radiodetector and the use of a large solid scintillation loop, have resulted in the broadening of the peaks and difference in the retention time of .53 minutes.

Considering the results from this reaction, the overall conclusion for, the use of tritium with the silane-Pd(0) as a method for labelling of peptides is not completely promising. Here a significant difference between the tritium reactions and the reactions performed with deuterium is shown. It is, at this point, not totally clear why this difference is observed when the silane chemistry is transferred to tritium.

If the tritiosilane and palladium(0) methodology is going to be an applicable method for labelling of peptides/proteins further investigations of this reaction have to be conducted.

First of all, it has to be clear, whether or not the triethyltritiosilane is formed in the reaction from lithium tritide. It is only proposed that this reagent is synthesised, no proof is though present for this formation. A good understanding of the reaction - mechanism and -kinetics would be preferred in the transfer of this deuterium chemistry to tritium. An investigation of the isotopic effect in the synthesis of the hydrido-silane could be beneficial for at least the first synthesis in this labelling reaction – in these tritium experiments analysis of intermediate products have not been possible.

Part II. Development of New Tritium Labelling Methods for Peptides.

Discussion; Scopes and Limitations for Tritium Labelling with Silanes and Palladium(0)

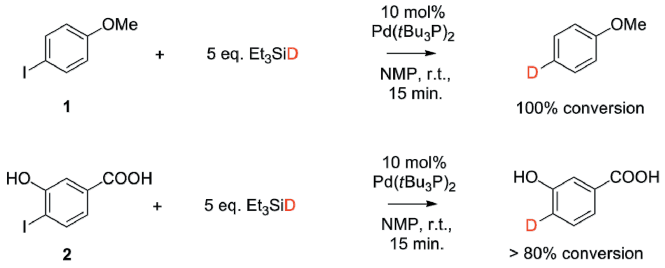
An investigation of the developed deuterio-triethylsilane method, used in the deuterium labelling of angiotensin-I, was continued on small molecules (defined here as molecules with Mw < 500 Da). These experiments were initiated due to the inconclusive results in the tritium labelling reactions of this peptide substrate.

Experiments performed with small iodinated aromatic molecules as the labelling substrates could possibly yield a better understanding of the reaction mechanism and potentially lead to a labelling method for sensitive substrates. The use of NMR as a analytical tool for reaction kinetics and identification of the reaction intermediates and products will give a clearer indication of the reaction. At this point a few simple reactions have been performed with HPLC as the analytical tool.

Recently, the deuterated triethylsilane (Et_3SiD) has become commercial available which will make screening of reaction conditions much easier. The preliminary results, presented in the following section, give a brief summary of the labelling reactions performed with the commercial reagents.

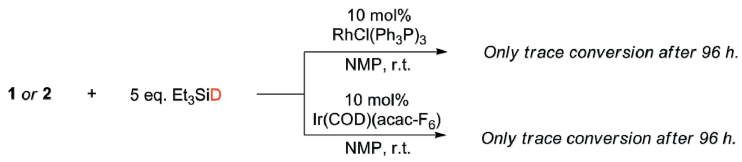
Preliminary results from deuteriodeioniation of small molecules with commercial triethyldeuteriosilane and bis(tri-tert.butylphosphine)palladium(0).

As hydridosilanes in combination with palladium catalysts have been reported to deiodinate simple aromatic compound, which was briefly described in the background. This important published[103, 105] reaction has a direct relevance to this ph.d. project, due to the idea of utilizing tritiated silanes as labelling reagents. Preliminary results in the investigation of this method, reactions with (recently available) commercial triethyldeuteriosilane, simple small molecules and three catalysts were tested. In the parallel reactions 5 equivalents of silane were used together with 10 mol% of catalyst (**scheme 14 and 15**).



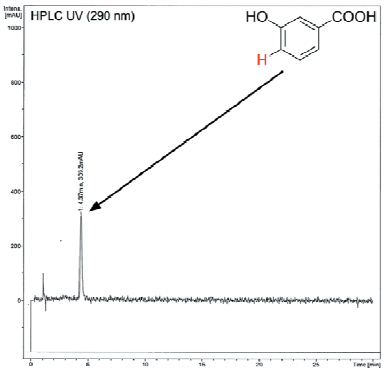
Scheme 14. The deuteriodeiodination reactions of *p*-iodo-anisole (1) and 3-hydroxy-4-iodobenzoic acid (2) using commercial Et_3SiD and palladium catalyst are shown.

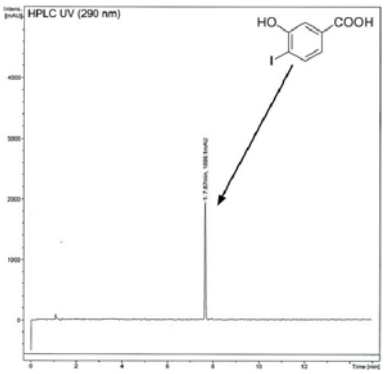
In the reaction using *bis*(tri-*tert.*butylphosphine)palladium(0) as catalyst, an immediately reactivity was observed. From the time adding the catalyst and until applying onto the HPLC for analysis (**figure 19 and 20**) all starting material was converted into the corresponding deiodinated products. This is in conflict with the literature presented results, because no silylated product was observed in these reactions. Instead these preliminary reactions, illustrates that very promising hydrodeiodination reactions can be performed fast at room temperature and with catalytic amounts of palladium catalysts. A rhodium(I) and a iridium(I) based catalyst were also tested in the same reaction, though only very little to no conversion were observed over a long period of time.



Scheme 14. The attempted deuterodeiodination reactions with Wilkinson's catalyst and a Ir(I) based catalyst are shown. No, or only a little, conversion were observed after 96 hours.

Part II. Development of New Tritium Labelling Methods for Peptides.





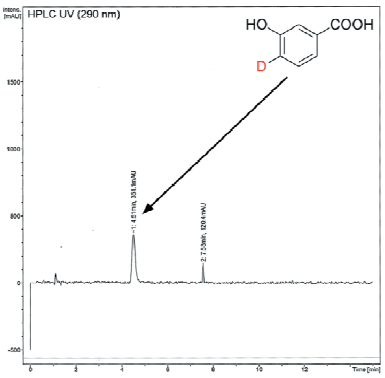
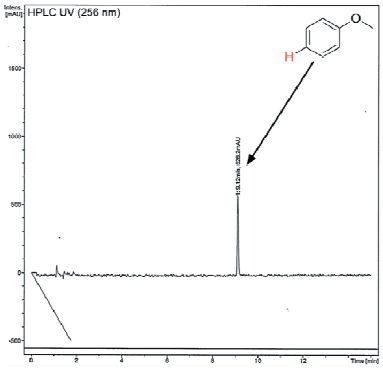


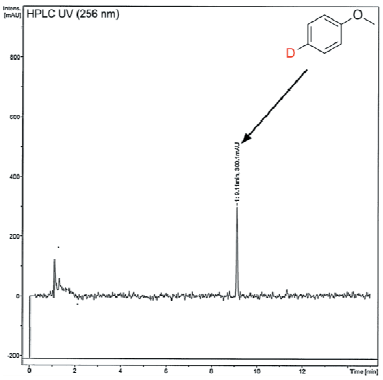
Figure 19. HPLC results from the reaction with 4-iodo-3-hydroxybenzoic acid, UV (290 nm) traces of:

(*top left*) 3-hydroxy-benzoic acid (Sigma-Aldrich). Rt. = 4.37 min.

(*top right*) 3-hydroxy-4-iodobenzoic acid (Sigma-Aldrich). Rt. = 7.67 min.

(*left*) products after 15 minutes reaction time. Deiodinated product, Rt. = 4.51 min.





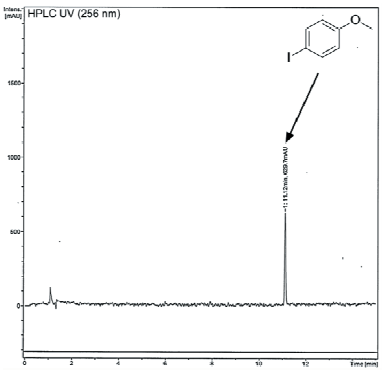


Figure 20. HPLC results from the reaction with *p*-iodo-anisole, UV (256 nm) traces of:

(*right*) anisole (Sigma-Aldrich). Rt. = 9.12 min.

(*bottom right*) *p*-iodo-anisole (Sigma Aldrich). Rt. = 11.12 min.

(*bottom left*) product after 15 minutes reaction time. Deiodinated product, Rt. = 9.15 min.

Procedure. In general, the solvents (THF and NMP) were degassed with bubbling of argon in dry round bottomed flasks.

A reagent solution of triethyldeuteriosilane (Et_3SiD) was prepared prior to the reactions. The reagent (~500 mg) was transferred to a sealed vial (20 ml), weighted and diluted to a concentration of 50 mg/ml (427 μmol/ml) with THF. This solution was added in the reagent amounts stated below in the parallel deuteration reactions.

The same procedure was used to prepare the catalyst solution. Under an atmosphere of argon *bis*(tri-*tert*.-butylphosphine)palladium(0) was added to a vial and dissolved in NMP to a concentration of 4.4 mg/ml (8.5 μmol/ml). The same regarding the rhodium and iridium catalyst (8.5 μmol/ml)

The reactions were performed (in parallel) in oven dried round bottomed flasks (50 ml) under argon with total substrate concentrations of 1 mg/ml for direct applications of aliquots to HPLC injections. 4-iodo-anisole (20 mg, 85 μmol) was added to the reaction flask in NMP (18 ml), then added the reagent solution (1 ml, 427 μmol, 5 eq.) and subsequently the catalyst solution (1 ml, 8.5 μmol, 0.1 eq.).

From the reaction mixture aliquots were collected at diffent times, transferred to a HPLC vial and subjected directly to analysis.

Conclusion. Preliminary experiments show promising results using hydridosilanes as labelling reagents for small molecules. The possibility of the chemical syntheses of tritiated silanes makes these reagents attractive for labelling of especially small molecules with sensitive functional groups. Immediate hydrodeiodination of the two substates were observed when 5 eq. of deuteriosilane were employed with homogeneous palladium. With use of rhodium and iridium catalysts a very slow reaction rate was observed but still product could be observed, in both reactions.

At this point, the identification of the hydrodeiodinated product is based on HPLC analysis alone. Further analyses, mass spectrometry and NMR will confirm that it is deuterium incorporation and also the degree of specific labelling. It is however slightly possible that it is a proton instead of deuterium labelling.

Proposed Future Experiments with Silanes and Palladium(0)

The method with use of hydrogen labelled silanes as reagents for labelling molecules with sensible functional groups is presented. This method is though not in-depth understood and still many experiments could be imagined for the optimisation of the reaction conditions.

For a better understanding of the reaction mechanism further investigations must be conducted. These experiments could be investigation of the reaction rate, the isotope effect or the tolerance towards a series of functional groups. Eventually this method could turn out to be an alternative labelling method for molecules with unsaturated bonds, benzaldehydes, carbonyls in general or aliphatic and aromatic halogen.

The silane utilised in these reactions are non-UV active and volatile. This gives rise to problems of detection by HPLC-UV and with volatile radioactivity.

Investigations of synthesis and reactivity of UV active and non-volatile tritiosilanes is proposed. One example could be dimethylphenylhydridosilane.

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Appendix I.

Selective deuteriodeiodination of iodinated tyrosine in peptides using deuterated triethylsilane and homogeneous Pd(0)

Selective deuteriodeiodination of iodinated tyrosine in peptides using deuterated triethylsilane and homogenous Pd(0)

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Abstract

In our efforts in the development of new reactions for the efficient labelling of peptides and proteins with tritium, we here wish to report the use of silanehydrides and homogenous Pd(0) mediation for the protio- and deuteriodeiodination of *o*-iodo-tyrosine of peptides at room-temperature.

Introduction

Tritium labelled compounds is an essential tool for ADME studies of new drug candidates and of new chemical entities in particular. Current methodologies for the efficient tritium-labelling of peptides include tritioacylation[1], spillover isotope exchange[2-3], olefin reduction[4-7] and hydrodehalogenations, *e.g.* using Pd/C and gaseous tritium[8-13]. The latter has been one of the preferred labelling methods for peptides whilst tritioacylation with N-succinimidyl [2,3-^{3}H] propionate presently is the reagent of choice for conjugation and labelling of free amino-groups

on both peptides and proteins[1,14-16]. In contrast, low or no isolated yields are often observed in reactions using heterogeneous palladium on supports and tritium gas in combination with larger peptides and proteins.[17] Plausible theories are that the substrate sticks or clots to the metal or support, or that the halogenated amino acid is not available at the surface of the metal-hydride and thus not accessible for the reaction with tritium at the metal surface. We therefore set out to investigate the potential of using homogeneous palladium as a labelling technique for hydrogen-halogen exchange of iodinated peptides. A homogeneous transition-state metal mediated reaction could possibly reduce both clotting and steric hindrance at the halogenated reaction site and would potentially lead to increased reaction rates and/or yields.

Homogenous catalysis has been extensively studied and has found a broad range of useful applications in synthetic chemistry. Numerous homogenous palladium(0) species have been reported to catalyse the cross-coupling reactions between organoboron[18] (Suzuki-Miyaura), organostannane[19] (Stille) and organosilane[20-22] (Hiyama) compounds and arylic or alkylic halogen substrates. Despite these achievements, only a few groups have applied the cross-coupling techniques for the functionalisation of peptides and proteins. With the use of water soluble phosphine ligands applicable for polar substrates Schmidtchen[23] and Ghadiri[24] were able to perform novel functionalisation onto peptides and proteins. These pioneering examples include the use of Pd(0) (Sonogashira) cross-coupling on peptides in aqueous solution. More recently, groups have begun to use homogenous palladium catalysis as a tool for peptide and protein modifications[25-28] including rhodium for olefine hydrogenation[29].

Literature examples of the use of silanes in cross-coupling reactions are limited in contrast to the much more frequent boron and stannate coupling reactions. Examples for the preparation and

use of tritiosilanes as reducing agents have been reported[30-31], and silanes have also been shown to reduce organic halides, mediated by palladium[32]. Yamanoi and Nishihara demonstrated the use of hydrosilanes and a palladium or rhodium catalysts for the silylation (Si-C) of aromatic halides[33-34]. Although the objective for their work was functionalisation of the arylic halogenide to give organnosilanes, they describe that the use of the bulky *tert.*-butylphosphine as ligand for the palladium cross-coupling reaction, almost exclusively defunctionalised and substituted the aromatic iodide to an aromatic hydrogen[35]. Interestingly this reduction was also observed when the Wilkinson catalyst ($RhCl(PPh_3)_3$) was employed in the same reaction. But changing one of the phosphine ligands with a carbonyl ligand ($RhCl(CO)(PPh_3)_2$) reversed the selectivity, now to give predominantly silylation[35].

The hydrides (protides, deuterides and tritrides) of boron, tin and silicon can be synthesized by nucleophilic substitution with the use of lithiumhydride and the corresponding halogenide (Scheme 2 and 3)[31,36]. The use of tritides ($T^{\delta-}$) as reducing agents has found its way to the radiochemistry laboratory and is today regularly used for radiolabelling of small molecules for drug development studies[37]. The weak hydride character derived from the small difference in electronegativity of silicon and hydrogen in silanes (Si-H) makes these reducing reagents promising in view of the multitude of functional groups often present in peptides.

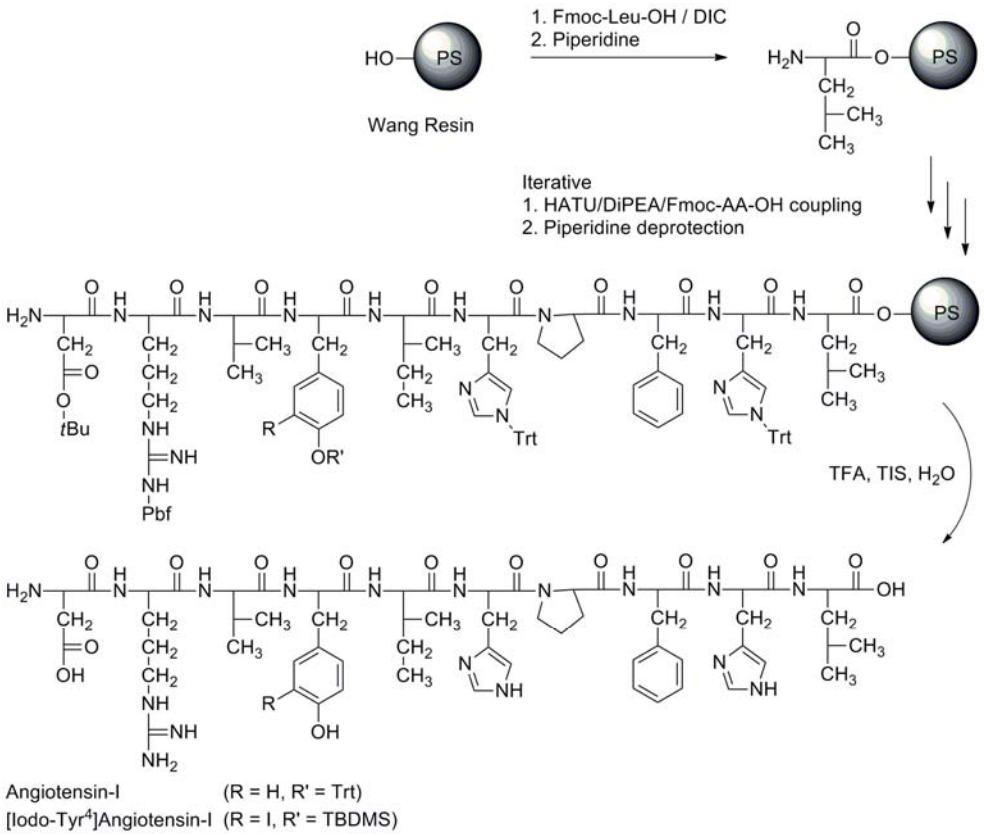
The objective of this work was development of efficient tritiation methods of an iodinated precursor by dehalogenation. To optimise the reductive hydrodehalogenation reaction the development work was performed using deuterium gas (D_2), resulting in deuterated silanes and subsequently deuterated peptides. The use of deuterium allows for optimisation without the use

of large amounts of tritium gas. When using deuterium, the analysis of deuterium incorporation may be achieved by mass spectrometry. In order to ensure that the developed chemistry will transfer smoothly to the intended tritium labelling methodology, deuterium labelling reactions were performed under typical radiolabelling (T) conditions starting with the synthesis of 160 micromoles lithium deuteride (theoretically 160 micromoles T equals 4.6 Ci of radioactivity) on a custom build tritium/deuterium manifold system (RC Tritec). The reactions were performed in a sequential manner as one-pot reactions without isolation of the intermediate products.

In the following, we present the preparation of deuteriosilanes which can be used as relatively weak hydride donors for exchange of iodide with a hydrogen isotope when using excess of silane. The reaction is proposed to be analogous to the reaction discovered by Hatanaka and Hiyama[38] with the use of silanes in coupling with homogenous palladium.

Results

The method has been optimized using the iodinated natural peptide Angiotension-I as a model-peptide. The iodinated substrate peptide [Iodo-Tyr^4]Angiotensin-I was synthesized on solid phase using standard Fmoc, HATU/DiPEA chemistry (SPPS) in manual synthesisers outlined on scheme 1. The reference [Tyr^4]Angiotensin-I material was obtained by splitting the resin before the tyrosine coupling. Please refer to the supporting information for experimental details. Angiotensin-I is a suitable model for the development of optimal reaction conditions. The ten-mer peptide contains a tyrosine residue at the 4th position and carries a variety of functional groups which could interfere with the use of a homogenous transition metal complexes and peptides.



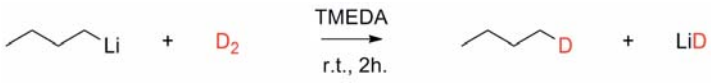
Scheme 1. Solid phase peptide synthesis of [Iodo-Tyr^4]Angiotensin-I for labelling and the reference compound (Angiotensin-I).

We first investigated the palladium mediated dehalogenation of the iodinated peptide using commercially available triethylsilane (Et_3SiH) and triethoxysilane ($(EtO)_3SiH$) in combination with the standard tetrakis(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) catalyst.

The commercial triethoxysilane ($(EtO)_3SiH$) proved to be an aggressive reducing agent corresponding with the high hydride character of the silane caused by the inductive effect of the

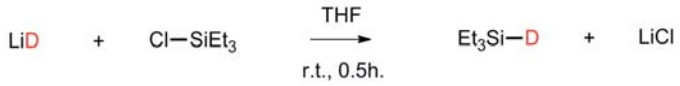
ethoxy substituents. After attempts to prepare deuterium labelled triethylsilane and triethoxysilane, the triethylsilane was selected as the hydride doner due to instability of EtO_3SiD in protic solvents.

The lithiumhydrides of hydrogen-isotopes are ready available when synthesized in micromolar scale with *n*-butyllithium (160 µmoles) and TMEDA (N,N,N',N'-tetramethylethylene-diamine) following litterature procedures[39]. Under an atmosphere of deuterium gas (600 – 800 mbar), we employed *n*BuLi (100 µL, 160 µmol) together with a slight excess of TMEDA (50 µL) as co-solvent for breaking the *n*BuLi hexamer into more reactive species. The formation of lithiumdeuteride (LiD) was complete after 1 - 2 hours as no further consumption of deuterium gas was observed (scheme 2).



Scheme 2. Formation of lithiumdeuteride.

After formation of the hydride, the volatiles (butane, solvents and TMEDA) was removed under vacuum and the dry lithiumdeuteride was used directly in the subsequent reactions.



Scheme 3. Synthesis of trialkylsilane.

The formation of deuteriotriethylsilane from LiD and triethylchlorosilane (Scheme 3) was investigated using GC with FID detection, and we found almost complete conversion within 30 minutes. The reactions were performed at room temperature in anhydrous THF and the formed deuteriosilane was used without further purification in the subsequent labelling reaction. Isolation and purification of the triethylsilane was attempted but with no success, as the silane is highly volatile, and is synthesized in less than 160 µmoles. The GC-FID chromatograms from the synthesis of deuteriotriethylsilane, starting material and reference triethylsilane are stacked and illustrated on figure 1.

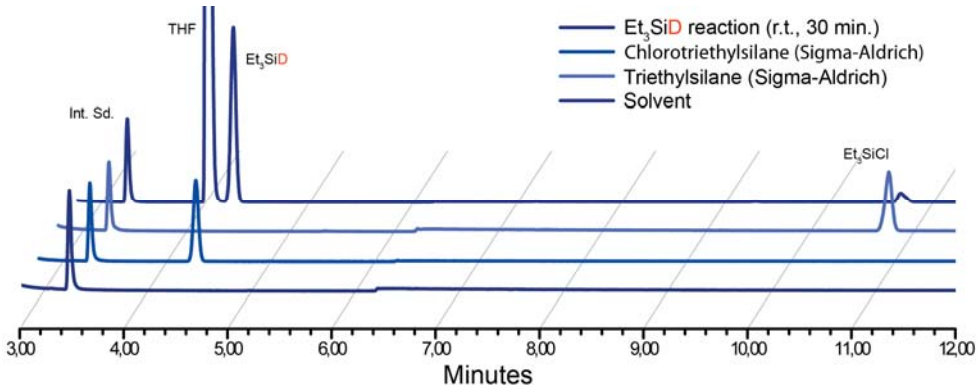


Figure 1. Stacked GC-FID chromatograms from silane synthesis.

The ^{1}H-NMR analysis of the Ei_3SiD product demonstrate the expected loss of a *J2* coupling between the α-ethylene and Si-H bond. Please refer to the supporting information for further details.

After having optimised the reaction conditions for the efficient formation of the triethylhydridosilane, attention was focused on the palladium mediated hydrodehalogenation reaction.

A number of phosphine ligands were investigated. The reactions were performed at room temperature and the conversion of starting material and formation of deuterated peptide was measured by mass spectroscopy (MS). A silylated byproduct was also formed and its formation monitored in MS. By tuning the steric properties of the ligand and the lewis base character of the phosphor the conversion and yield could be optimized. Please refer to table 1, for a selected list of tested phosphine ligands.

Table 1: Optimisation of phosphine ligand

Reaction nr.	Phosphine	Reagent amount	Conv. *	Products*
-	No Phosphine	135 µmole Et_3SiD	100%	100% D[C]
-	DPPP	256 µmole Et_3SiD	N/A[A]	Traces[A]
-	PCy_3	135 µmole Et_3SiD	0%	0% D
-	DPPF	160 µmole Et_3SiD	10%	30% H/ 70% D[B]
1	PPh_3	1100 µmole Et_3SiD	15%	100% D
2	PPh_3	320 µmole Et_3SiD	14%	100% D
4	P(*o*-furyl)$_3$	270 µmole Et_3SiD	69%	28% D + 38% D/TES
9	P(*t*Bu)$_3$	160 µmole Et_3SiD	100%	36% D + 64% D/TES

*Conversion of [Iodo-Tyr^4]Angiotensin-I and analysis of products formed in the optimisation reactions were determined by mass-spectrometry where D means [D-Tyr^4]Angiotensin-I and D/TES corresponds as [D-Tyr^4]Angiotensin-I functionalised with a triethylsilyl group. Reaction number refers to the supplemental information.
[A] Several unidentified products seen in MS.
[B] Estimated ratio of H and D by MS.
[C] Reaction with $Pd(OAc)_2$, instantly formation of palladium black, inhomogenous reaction. A full conversion and labelling observed by MS with Pd adducts.

Besides the phosphines listed in table 1, we also tested the non-phosphorus DABCO, but the reaction turned black instantly, possibly due to silane mediated reduction of Pd(II) to palladium black. This was also found in the reaction with $Pd(OAc)_2$ and absence of a phosphine ligand. Due to the apparent non-homogeneity of these reactions, they were not investigated further.

It was found that the bulky and electron donating tri-*tert*.-butyl phosphine ligand (reaction 9) gave the best conversion of the iodinated peptide to deuterated product. It should be emphasised that this ligand is very sensible to oxidation, and that the reactions must be performed under inert atmosphere (argon).

After having identified the optimal ligand, attention was turned to the necessary quantity of Et_3SiD. It was found that with an increase of the amount of prepared silane from 50 to 135 µmole, the conversion of substrate went from only traces of deiodinated product to complete deiodination. Results are summarized in table 2, where the quantity of the deuterated silane is calculated on the basis of the quantity of *n*BuLi used in the preparation lithium tritide, as no method was available for its direct quantitation.

Table 2: Optimisation of deuteriosilane and palladium quantity

Reaction nr.	Et_3SiD / Pd	Conv.[§]
5	50 / 10 µmole	1%*
6	95 / 10 µmole	22%*
7	135 / 10 µmole	74%*
8	135 / 20 µmole	100%

§ Conversion of was determined by MS.
* Clear palladium adducts was observed in MS.

The observed dependence on the quantity of the silane may be explained by the simple fact that the reaction involves handling of very small quantities of highly air sensitive materials (BuLi and LiT).

The results in Table 2 also show that the use of larger amounts of the palladium catalyst results in increasing yields. It should be noted, that an excess of catalyst to iodinated peptide is

employed. The handling of catalytic amounts (in the nanomolar range) of the palladium catalyst is difficult and not an important parameter in these experiments. The successful formation of deuterated (or tritiated) peptide is the ultimate goal of this work. On the other hand a suitable balance must be found, as the use of a very large excess of palladium may pose a problem in its the later removal after labelling reaction.

As shown in table one, the reaction also resulted in the formation of an undesired byproduct from triethylsilyl (TES) conjugation with the deuterated peptide. Further experiments demonstrated its effective elimination through treatment with trifluoroacetic acid (TFA) or tetrabutylammonium fluoride (TBAF). The formation of TES conjugated angiotensin can be explained by a reaction between hydroxyl groups on the deuterated or iodinated peptide and unreacted chloro-silane or the potentially formed iodo-silane.

Table 3 summarizes the effect of TBAF added at the end of reaction.

Table 3: Optimisation of reaction time and use of fluoride

Reaction nr.[§]	TBAF / L	Time	Conversion	D/TES-product[*]	D-product[*]
2	0 eq. / PPh_3	3 h	14%	0%	14%
3	1 eq. / PPh_3	3 h + 0.5 h	30%	0%	30%
9	0 eq. / P*t*Bu$_3$	3 h	100%	64%	36%
10[A]	1 eq. / P*t*Bu$_3$	1 h + 0.5 h	11%	0%	11%
11[B]	1 eq. / P*t*Bu$_3$	3 h + 0.5 h	100%	0%	100%

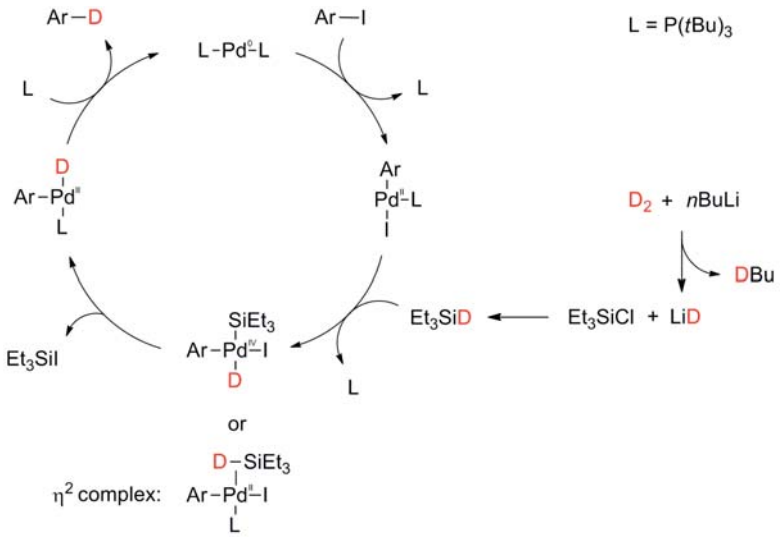
§ Reaction nr. refers to the supplementary information.
* The ratio between products ([D-Tyr^4]-Angiotensin-I and triethylsilyl-[D-Tyr^4]-Angiotensin-I) and substrate ([iodo-Tyr^4]-Angiotensin-I) was determined by mass-spectrometry. Amount of used Et_3SiD was 160 µmole in reaction nr. 3, 9, 10 and 11, and 320 µmole in reaction nr. 2.

It was found that the addition of TBAF to the reaction mixture seems both to eliminate the formed TES-product and to accelerate the deuteration reaction (see reaction 2 & 3). The mechanism for this was although not investigated further.

With these optimisations, we were able to perform the efficient deuteration of iodinated angiotension starting from deuterium gas in just one day.

Conclusion. This study concerns the use of silanes the in selective dehalogenation of an iodinated model peptide using homogenous palladium catalysis. The reaction uses mild reducing conditions and has been shown to tolerate several peptide functional groups such as *e.g*. amides, carboxylicacids and phenol. We have demonstrated that it was possible to label an iodinated model peptide (Angiotensin-I) with deuterium using this chemistry. Although the exact mechanism for this deuteriodeiodination reaction is not clear, we propose that the reaction could occur through the generally accepted palladacycle illustrated in scheme 3. The tri-*tert.*-butylphosphine ligand is well known to generate low-ligated and very reactive palladium species[40-43] which after oxidative addition into the aromatic iodine bond could undergo transmetallation with the synthesised deuteriosilane.

We suggest a reaction mechanism as described below (Scheme 4). This is in line with a recent publication showing that the addition of base to the system results in formation of C-Si silylated products.[44].



Scheme 4. Proposed mechanism for the deuteriodeiodination of an iodinated aromatic compound.

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Supporting Information Available: Experimental procedures, Material and Methods, Mass-Spectra, HPLC-chromatograms, NMR data and GC-FID.

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Supporting Information

for

"Selective deuteriodeiodination of iodinated tyrosine in peptides using deuterated triethylsilane and homogenous Pd(0)."

Martin Holst Friborg Pedersen and Lars Martiny.

Illustration and figure index.

Illustration 1A. Picture of the deuterium/tritium manifold system.
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General Information

All reactions were performed on a custom build deuterium or tritium manifold system (Illustration 1A and 1B) manufactured by RC Tritec AG, Switzerland. Deuterium gas was supplied onto the manifold and all chemical manipulations were performed in glassware and in amounts analogous to working with tritium. Reagents and solvents were used as received from Sigma-Aldrich without further purification. Catalysts, phosphines and compounds unstable to oxygen where handled under an argon atmosphere in a custom made glovebox. Solvents where deoxygenised by ultra sonic irradiation followed by thorough bubbling with argon.

Illustration 1A. Picture of the deuterium/tritium manifold system.

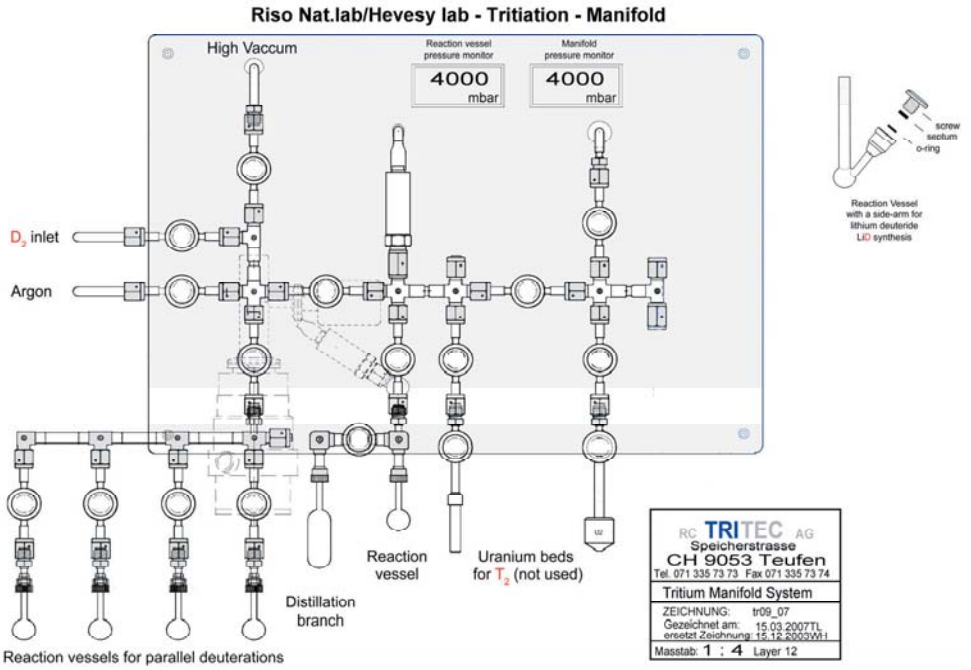


Illustration 1B. Schematic drawing of the deuterium/tritium manifold system and reaction vessel used for lithium deuteride (LiD) synthesis.

Instruments

Gas Chromatography was performed on a Varian 3900, with manual injection and equipped with a FactorFour™ (Fused Silica) capillary column (VF-200ms, 30m x 0.32mm ID, DF=1.0, Varian) and a FID detector. Mass spectrometry was performed on a Bruker Esquire 4000 iontrap (IT) equipped with a electrospray ionization (ESI) interface. Preparative rp-HPLC was performed on a Knauer Smartline. (Components: Manager 5000, Pump 1000, UV Detector 2500) with the C-18 column at ambient temperature (20^{o}C). Analytical rp-HPLC was performed on a Waters Breeze system, Pump – Waters 1525, Binary Pump. UV Detector – Waters 2787, Dual λ Absorbance Detector with the C-18 column at ambient temperature (20^{o}C). Ultra purified water was supplied from a Sartorius arium® 611 water system. Lyophilisation of products were performed on a Hetosicc freezedrier, Heto.

Materials

Sep-Pak Cartridges; Sep-Pak® Plus C-18 Cartridges. Syringe Filter; Whatman Glass Microfiber GF/C, 25 mm diameter, 1.2µm pore size. Syringes and Needles; Single use Syringes (1, 2, 5 mL) and disposable Needles (Ø 0.08 x 120 mm, 21 G x 4¾", Sterican®), Braun.

Solvents and Reagents

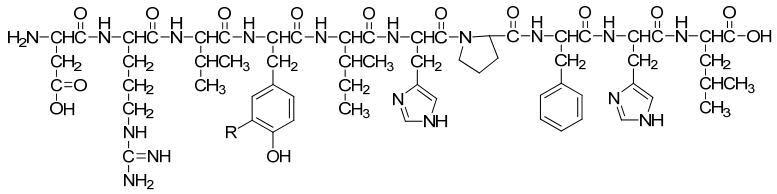
Chemicals where used as received from the supplier. Moisture sensitive compounds was stored in at 5°C in desiccators if necessary. Deuterium gas (D_2) (97.9%) was supplied from AGA. N,N,N',N'-Tetramethylethylene-diamine (TMEDA, 99%), n-Butyllithium (1.6 M in hexanes), N,N-Dimethylformamide (DMF, anhydrous 99.8%), Tetrahydrofuran (anhydrous, 99.9%, inhibitor-free), chlorotriethylsilane (TESCl, 99%), tetrabutylammonium fluoride (TBAF, trihydrate, 97%), palladium(II) acetate (99.9%+ metal basis), tri-*tert.*-butylphosphine (1M in toluene) where all supplied by Sigma-Aldrich.

The Fmoc protected amino acids where used as received from supplier GL Biochem (Shanghai) Ltd.(Fmoc-His(Trt)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ile-OH, Fmoc-Tyr(Trt)-OH, Fmoc-Val-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(*t*Bu)-OH). Fmoc and TBDMS protected 3-iodo-tyrosine was synthesised prior to acylation.

Experimental details

[Iodo-Tyr^4]Angiotensin-I and [H-Tyr^4]Angiotensin-I

The peptide substrate and reference compound were synthesised on solid support with standard Fmoc chemistry using HATU/DiPEA as the activation protocol. The synthesis of the two peptides was performed by splitting resin (20:1) after the isoleucine coupling.



Loading Protocol: 500 mg Wang resin (loading approx. 1 mmol / g.) was placed into a manual peptide synthesis glass-reactor (10 ml, Peptides International Inc.) and loaded with the first amino acid with the symmetrical anhydride procedure.

Symmetrical anhydride procedure: Fmoc-Leu-OH (1767 mg, 5 mmol) was agitated with DIC (391 µL, 2.5 mmol) in dry DCM for 20 minutes. The solvent was removed in vacuum and the anhydride was redissolved / suspended in 4 mL DMF. This was added to the resin with a catalytic amount of DMAP and the reactor was left rotating for 5 hours. After filtration the resin was rinsed 4 times with DMF and 4 times with DCM before it was dried down.

Fmoc Deprotection: The resin loaded with the Fmoc protected amino acid was swelled with DMF and subsequently treated with 20% piperidine in DMF for Fmoc deprotection. The resin was washed twice with DMF then twice with DCM before performing the Kaizer test and the peptidyl resin was swelled in DMF before the acylation step.

Acylation: In each coupling-step 4 eq. of Fmoc protected amino acid and 3.9 eq. HATU was mixed with a minimum amount of DMF for total dissolution. 8 eq. of DIPEA was added and the

solution was subsequently transferred to the N-deprotected peptidyl resin. The resin was agitated gently by rotation for 30 – 40 minutes before the remaining acylation agents was washed off with 4 times DMF and 2 times DCM. Kaizer test was performed after each acylation step, to ensure complete coupling.

Cleavage: A mixture of 90% trifluoroaceticacid (TFA), 5% triisopropylsilane (TIS), and 5% H_2O (4 ml) was applied to the resins for 3 hours for cleavage of peptide and protecting groups. After completion, the resin was extracted with 10 times of pure water. The residual TFA was gently removed under reduced pressure and finally the crude product was lyophilized.

[Iodo-Tyr^4]Angiotensin-I

The crude product was dissolved in water (5 mL) and gently washed with ethyl acetate (2 x 5 mL). The organic phase was discarded and the aqueous solution containing the peptide was purified on a preparative HPLC system with UV detection set at 280 nm. Column: C18-Knauer. (250mm x 20 mm, 5 µm Eurosphere-100) Flow: 10 mL/min. Eluents: A) 5% acetonitrile, 0.1% TFA, purified H_2O. B) 99.9% acetonitrile, 0.1% TFA.
Gradient: (0 min. - 4 min., 0% B); (-17 min., 35% B); (17 - 25 min., 100% B); (25 - 28 min., 100% B);(- 29 min., 0% B); (29 - 33 min, 0% B). The product ([Iodo-Tyr^4]Angiotensin-I) was collected after 17.4 min and lyophilised to yield a white flocculent powder: 319 mg.

The purified peptide was analysed on an analytical rp-HPLC system equipped with UV detection at 280 nm. Column: Phenomenex® Luna PFP(2) (250 mm x 4.6 mm, 5 µm). Flow: 1 ml/min. Eluents: A) 5% acetonitrile, 0.1% TFA, purified water. B) 99.9% acetonitrile, 0.1% TFA.
Gradient: (0-2 min, 0% B); (-40 min, 100% B); (40-42 min, 100% B); (-44 min, 0% B);(44-50 min, 0% B). The substrate ([Iodo-Tyr^4]Angiotensin-I) eluted at 17.4 min. Purity was determined by HPLC-UV to be: 97% (figure 1, top). The identity was confirmed by mass spectrometry illustrated on figure 1, bottom.

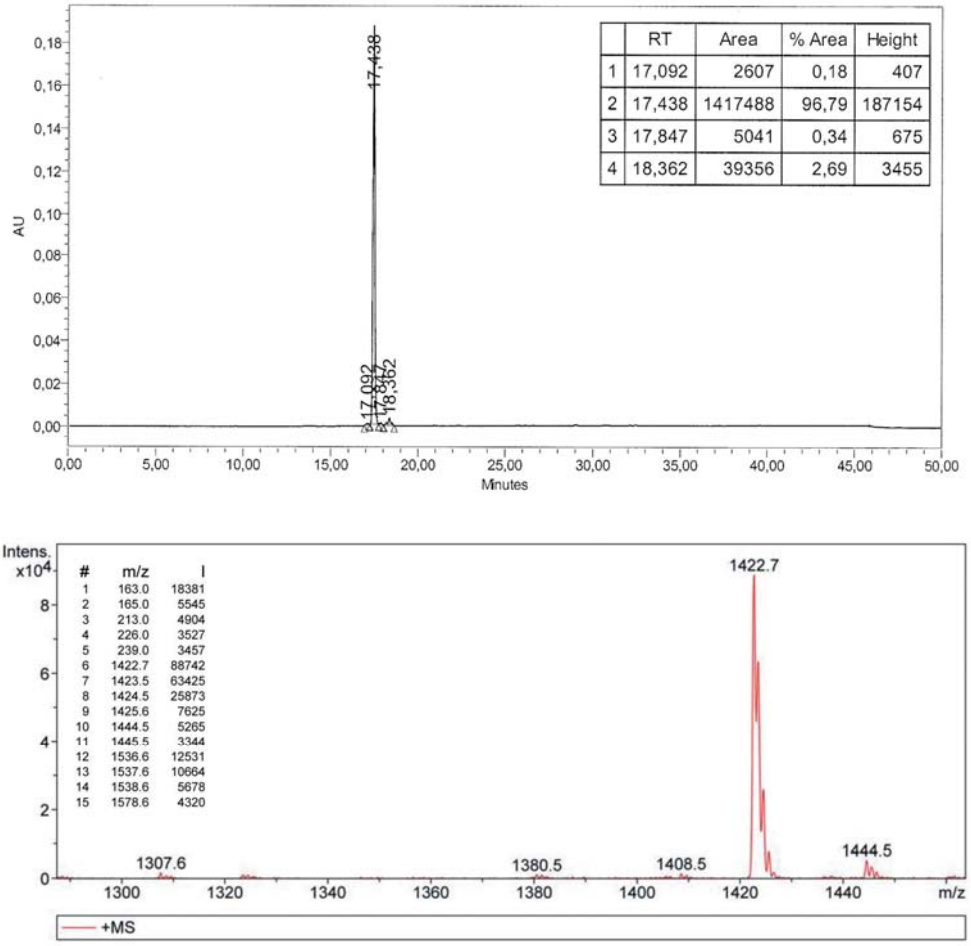


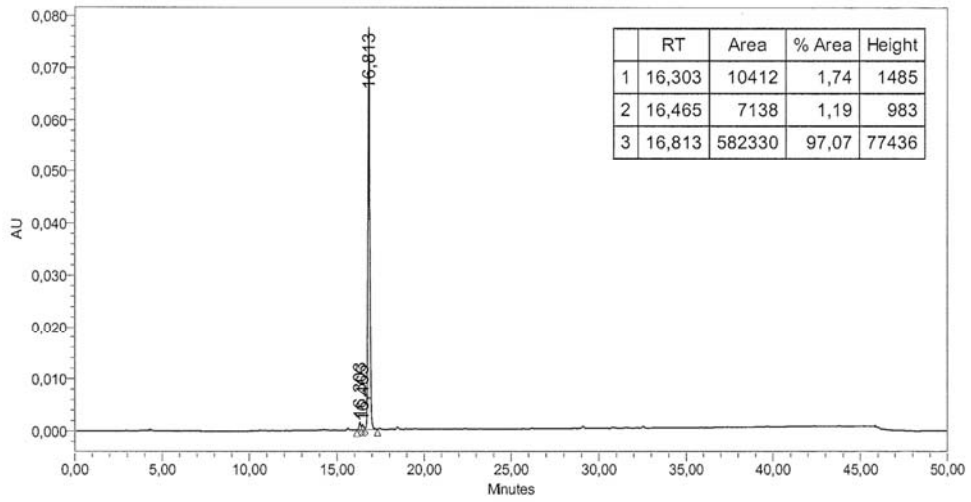
Figure 1. (*top*) HPLC chromatogram. (*bottom*) MS of [Iodo-Tyr^4]Angiotensin-I ($C_{62}H_{88}IN_{17}O_{14}$, M_w = 1421.6) after purification. m/z = 1422.7 ($M + H^+$), m/z = 1444.5 ($M + Na^+$).

Angiotensin-I

The crude product was dissolved in water (5 ml) and washed with 2 x EtOAC (5 ml). The organic phase was discharged and the water including peptide was applied onto preparative HPLC for purification detection at 280 nm. Column: C18-Knauer. (250mm x 20 mm, 5 um Eurosphere-100) Flow: 10 ml/min. Eluents: A) 5% acetonitrile, 0.1% TFA, H_2O. B) 99.9% Acetonitrile, 0.1% TFA. Gradient: (0-4 min, 0% B); (-17 min, 35% B); (-25 min, 100% B); (25-28 min, 100% B); (-29 min, 0% B); (29-33 min, 0% B). The desired product (Angiotensin-I) was collected after 17.4 min and lyophilised to yield a white flocculent power: 16 mg.

The product was analysed on analytical rp-HPLC equipped with column: Luna PFP(2). (250mm x 4.6 mm, 5 µm) Flow: 1 ml/min. Eluents: A) 5% acetonitrile, 0.1% TFA, H_2O. B) 99.9% acetonitrile, 0.1% TFA.

Gradient: (0-2 min, 0% B); (-40 min, 100% B); (40-42 min, 100% B); (-44 min, 0% B); (44-50 min, 0% B). The desired product (Angiotensin-I) eluted at 16.8 min. Purity determined by UV detection: 97% (figure 2, top) and the identity of the product was confirmed by MS (figure 2, bottom).



	RT	Area	% Area	Height
1	16,303	10412	1,74	1485
2	16,465	7138	1,19	983
3	16,813	582330	97,07	77436

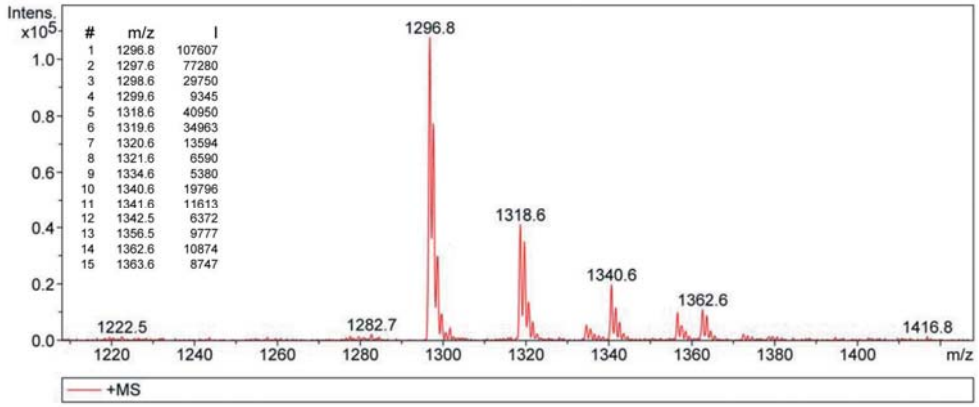


Figure 2. (*top*) Analytical HPLC of Angiotensin-I after purification. (*bottom*) MS of Angiotensin-I ($C_{62}H_{89}N_{17}O_{14}$, M_w = 1295.7) after purification. m/z = 1296.8 ($M + H^+$), m/z = 1318.6 ($M + Na^+$), m/z = 1340.6 ($M + 2 \times Na^+$), m/z = 1362.6 ($M + 3 \times Na^+$).

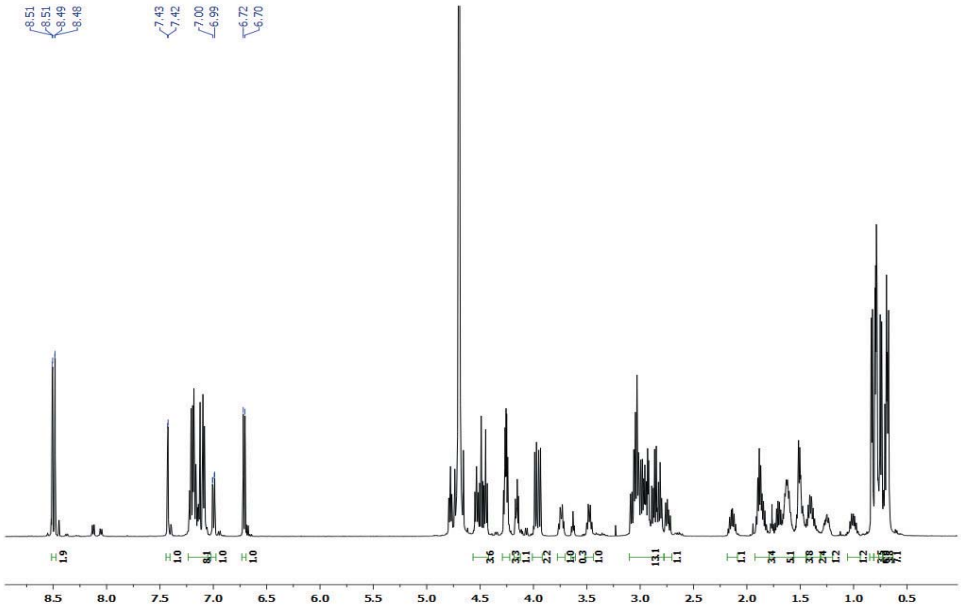


Figure 3. (^{1}H-NMR, D_2O, 500 MHz) [Iodo-Tyr^4]Angiotensin-I.

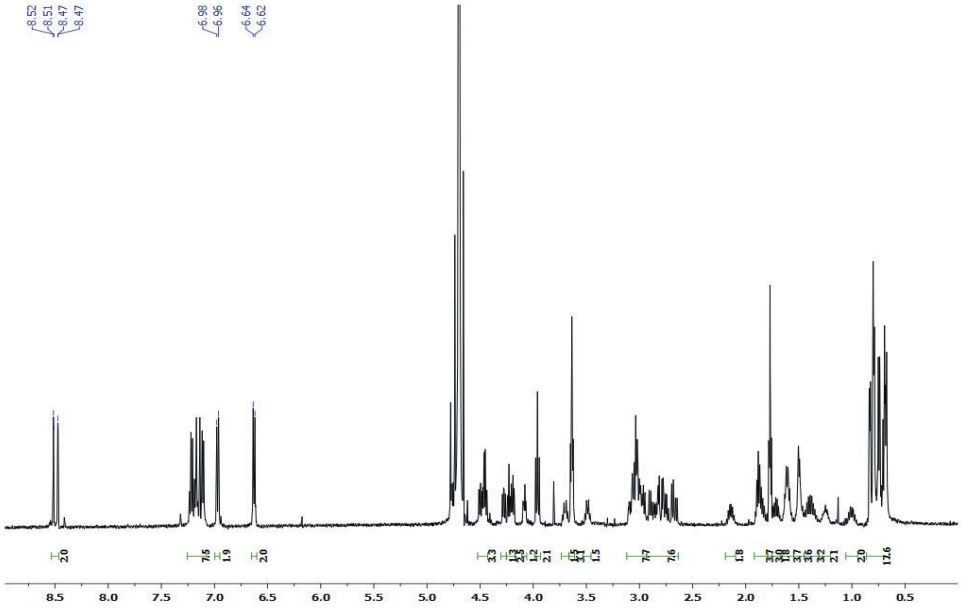
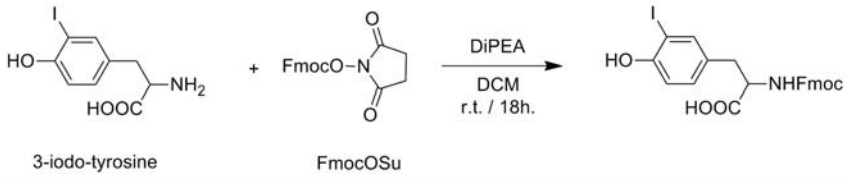


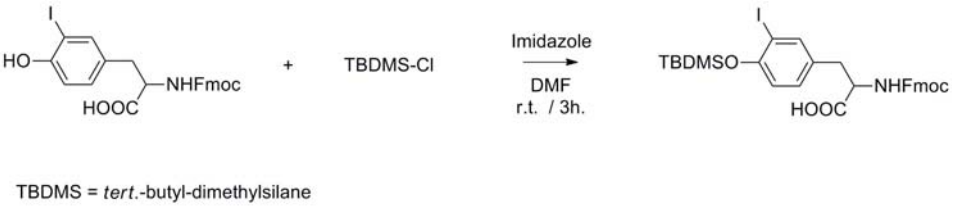
Figure 4. (^{1}H-NMR, D_2O, 500 MHz) Angiotensin-I

N-Fmoc-3-iodo-O-TBDMS-Tyrosine



Scheme 1. Fmoc protection of 3-iodo-tyrosine

In a 100 ml round-bottomed flask 3-Iodo-tyrosine (2164 mg, 7.05 mmol) and Fmoc-OSu (2607 mg, 7.73 mmol) was mixed with DCM (30 ml) to give a white suspension. Diisopropylethylamine (2.58 ml, 14.80 mmol) was added and the suspension allowed to react overnight at room temperature. A solution of $KHSO_4$ (2M, 2 mL) was added to the reaction mixture and the resulting suspension was stirred vigorously for 1 hour (a white precipitate was formed almost instantly). The product was isolated by filtering through a medium frit, washed with purified water (2 x 20 mL) and dried in vacuum before analysed. Yield: 2.96 g. (79%). Product showed one compound on TLC and ESI-MS confirmed a mass of m/z = 551.9 ($M + Na^+$) and m/z = 1079 ($2xM + Na^+$). The product was used in the following reaction without further purification.



Scheme 2. TBDMS protection of N-Fmoc-3-iodo-tyrosine

Fmoc-(3-iodo)-tyrosine (616 mg, 1.16 mmol) was dissolved in DMF (10 mL) and imidazole (316 mg, 4.66 mmol) was added. TBDMS-Cl (2.5 eq., 438 mg, 2.91 mmol) was added to the solution and the reaction was stirred for 3 hours for completion. The reaction mixture was added to a solution of $KHSO_4$ (25 mL, 0.4 M) and subsequently extracted with EtOAc (3 x 30 mL). The combined organic phases were evaporated and dried over night under vacuum giving the

crude product as a slightly yellow sticky solid (823 mg).

The crude product was purified using flash-chromatograpy with heptane, ethylacetate and methanol as eluents. The product eluted over many fractions and those were collected and evaporated to give the final product 623 mg (84%) which was used as the monomer in the synthesis of the iodinated angiotensin-I peptide. ESI-MS showed m/z = 666.2 ($M + Na^+$), m/z = 682.2 ($M + K^+$) and m/z = 1309.2 ($2 \times M + Na^+$).

General procedure for the deuterium labelling using tri-*tert.*-butylphoshine, palladium acetate and deuteriotriethylsilane.

Lithiumdeuteride (LiD).

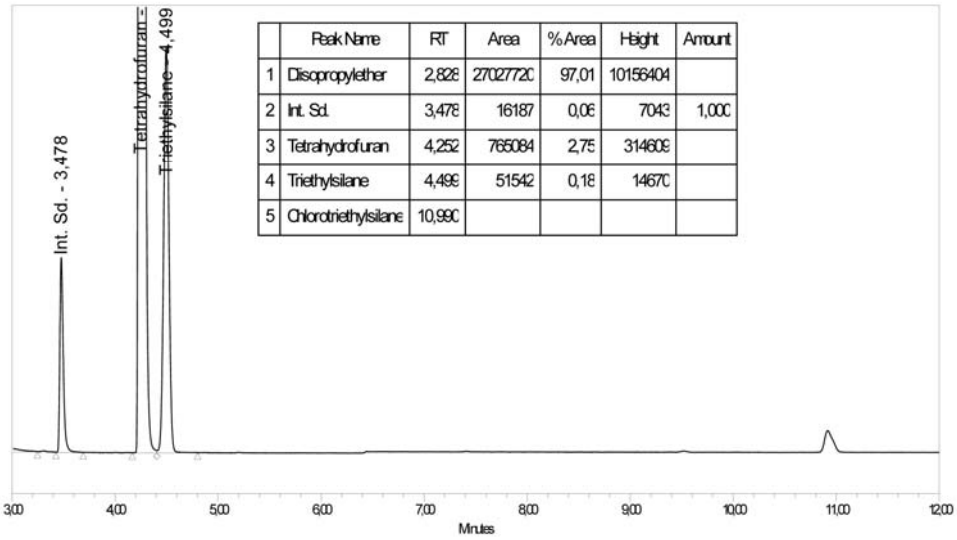
A 3 mL round bottomed flask with a side arm (for the addition of reagents) and magnetic-bar was mounted on the manifold system, evacuated to below $5x10^{-3}$ mbar and flame dried. The flask was flushed with deuterium gas and evacuated repeatedly to ensure an anhydrous and oxygen free reaction vessel. Finally the flask was loaded with deuterium gas to a pressure of 800 mbar. TMEDA (50 µL) and *n*BuLi (100 µL, 1.6M in hexane) were added with a syringe through the septum and within 5 - 10 minutes a white precipitate of LiD was formed. The reaction was stirred and allowed to react for 2 hours. Subsequently the flask was cooled with liquid nitrogen and the deuterated butane (byproduct), solvent (hexane) and TMEDA was removed under reduced pressure. The formed LiD was thoroughly dried under vacuum until dryness (white and flaky LiD, theoretical amount of 160 µmole, 1.44 mg). Finally the reaction vessel was flushed with argon at 800 mbar to ensure an inert atmosphere before the following reaction.

Deuterio-triethylsilane (Et_3SiD).

Under an atmosphere of argon a solution of chlorotriethylsilane (0.5M, 2 mL) in dry THF was prepared in a just prior to use. This solution (300 µl, 150 µmol of TESCl) was added to the lithiumdeuteride in the flask mounted on the manifold. The suspension was allowed to react for 1 hour at room temperature under stirring. The reaction was confirmed by GC-FID to be near completion after 30 minutes. (GC: $Rt_{Si\text{-}(D/H)}$ = 4.50 min.; $Rt_{Si\text{-}Cl}$ = 10.99 min.). GC-chromatograms are illustrated on figure 5 – 7, ^{1}H-NMR on figure 8 and 9.

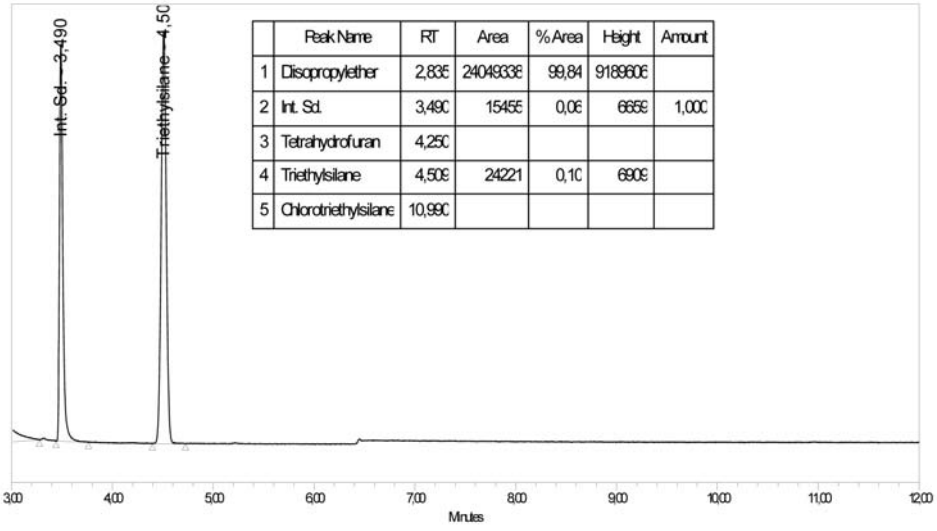
CG-FID specifications: Column Oven Temperature Program (0 - 3 min., 40 °C); (4 °C/min. until 100 °C); (40 °C/min until 260 °C); (3 min. at 260 °C); injector temperature: 220 °C.

The resulting suspension of deuteriotriethylsilane and LiCl in THF was used directly in the labelling step.



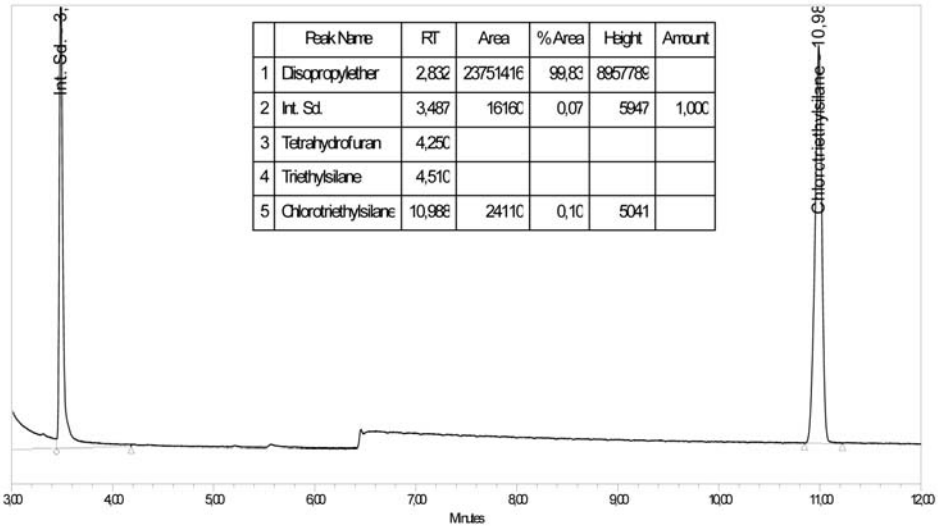
	Peak Name	RT	Area	% Area	Height	Amount
1	Diisopropylether	2,828	2702772	97,01	10156404	
2	Int. Std.	3,478	16187	0,06	7043	1,000
3	Tetrahydrofuran	4,252	765084	2,75	314609	
4	Triethylsilane	4,499	51542	0,18	14670	
5	Chlorotriethylsilane	10,990				

Figure 5. GC-FID chromatogram of deuteriotriethylsilane (Et_3SiD) after 30 minutes reaction time.



	Peak Name	RT	Area	% Area	Height	Amount
1	Diisopropylether	2,835	24049338	99,84	9189606	
2	Int. Std.	3,490	15455	0,06	6659	1,000
3	Tetrahydrofuran	4,250				
4	Triethylsilane	4,509	24221	0,10	6909	
5	Chlorotriethylsilane	10,990				

Figure 6. GC-FID chromatogram of triethylsilane (Et_3SiH) as received from Sigma-Aldrich.



	Peak Name	RT	Area	% Area	Height	Amount
1	Diisopropylether	2,832	23751416	99,83	8957789	
2	Int. Std.	3,487	16160	0,07	5947	1,000
3	Tetrahydrofuran	4,250				
4	Triethylsilane	4,510				
5	Chlorotriethylsilane	10,988	24110	0,10	5041	

Figure 7. GC-FID chromatogram of chlorotriethylsilane (Et_3SiCl, Rt. = 10.98) as received from Sigma-Aldrich.

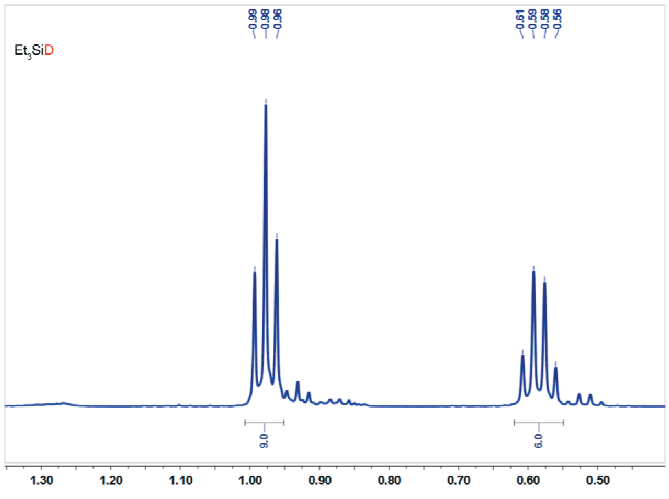


Figure 8. (^{1}H-NMR, $CDCl_3$, 500 MHz) Deuteriotriethylsilane (Et_3SiD)

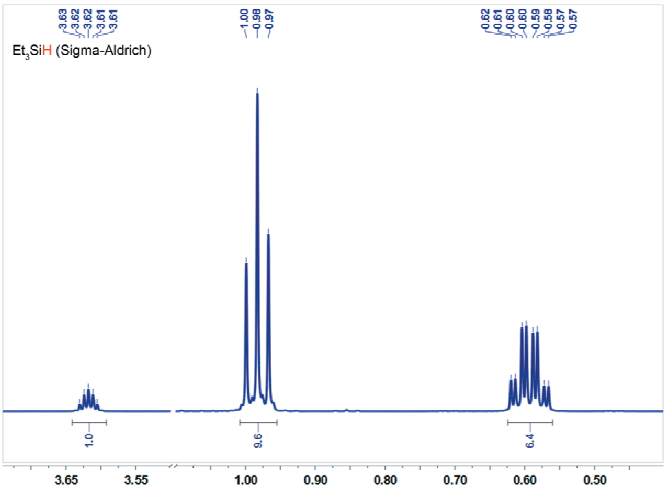


Figure 9. (^{1}H-NMR, $CDCl_3$, 500 MHz) Triethylsilane (Et_3SiH) as received from Sigma-Aldrich.

Deutero-deiodination of [Iodo-Tyr^4]Angiotensin-I. (Reaction 11)

A solution of the palladium catalyst was prepared prior to use under argon; Palladium acetate was added tri-*tert*.-butylphosphine (5 eq., 1M in toluene) and allowed to react at room temperature for 30 minutes (a change from deep orange to bright yellow coloured solution is observed).

In a vial [Iodo-Tyr^4]Angiotensin-I (2.9 mg, 1.56 µmoles) was dissolved in thoroughly deoxygenised DMF (600 µL). To the substrate vial the palladium catalyst (20 µmole, 100 µL) was added. It was allowed to react in 10 minutes before all was transferred to the reaction vessel containing the synthesised deuteriotriethylsilane, leaving a pale yellow homogenous reaction mixture. After 3 hours of reaction time 1 eq. of TBAF (160 µmole, fluoride) was added to the reaction and after 30 minutes the workup of product was performed.

A Sep-Pak® Plus C18 Cartridge (360 mg) with a 0.45 µm Wattmann CF-C filter was preconditionized with Ethanol (5 ml) for 1 min. then washed with distilled water (5 ml). Semi-purification of product was performed by mixing the reaction with 5 times the volume of purified water (5 ml) and filtering the preticipate through the filter and Sep-Pak. The filter and Sep-Pak was washed with ultra-purified water (5 ml) for the removal of salts and polar compounds. The filter was disposed and the peptide eluted from the Sep-Pak using a mixture of 80% MeCN, 20% Water and 0.1% TFA (5 ml).

Products and conversion of substrate was analysed using MS (ESI-IT) and HPLC. The final purification was performed by preparative HPLC with the following configuration.

Preparative HPLC: Column: C18-Knauer. (250mm x 20 mm, 5 um Eurosphere-100) Flow: 10 ml/min. Eluents: A) 5% acetonitrile, 0.1% TFA, H_2O. B) 99.9% acetonitrile, 0.1% TFA.
Gradient: (0-1 min, 0% B); (-25 min, 50% B); (-30 min, 100% B); (30-32 min, 100% B);(-33 min, 0% B); (33-34 min, 0% B). The desired product ([Tyr$_4$-^{2}H]Angiotensin-I) was collected in the interval 21-21.6 min, then lyophilized to give an isolated yield: 1.19 mg, app. 0.68 µmoles, 44%. The product was identified by rp-HPLC (figure 10) and MS (figure 11).

Analytical-HPLC: Column: Luna PFP(2). (250mm x 4.6 mm, 5 µm) Flow: 1 ml/min. Eluents: A) 5% Acetonitrile, 0.1% TFA, Purified Water. B) 99.9% Acetonitrile, 0.1% TFA.

Gradient: (0-2 min, 0% B); (-40 min, 100% B); (40-42 min, 100% B); (-44 min, 0% B);(44-50 min, 0% B). The desired product ([Tyr_4-^{2}H]Angiotensin-I) was eluting at 16.7 min. Purity: 96.5%.

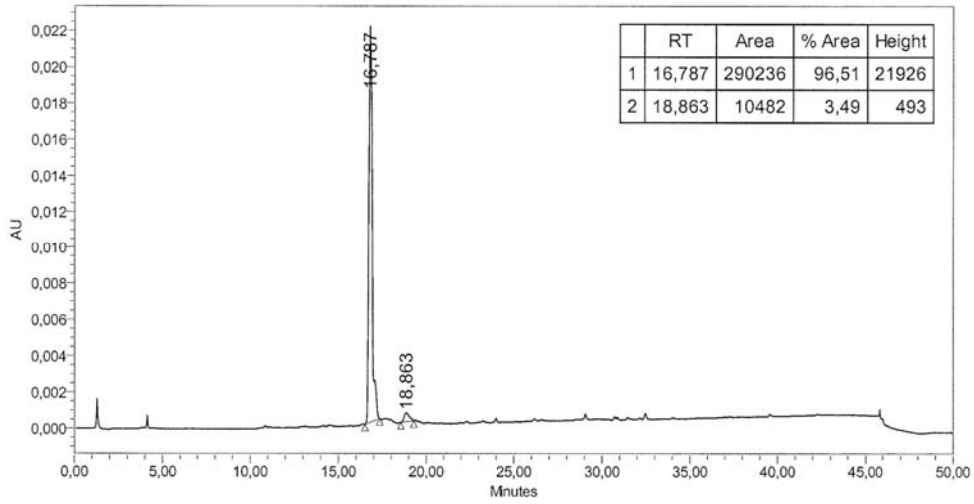


Figure 10. HPLC chromatogram of [D-Tyr^4]Angiotensin-I.

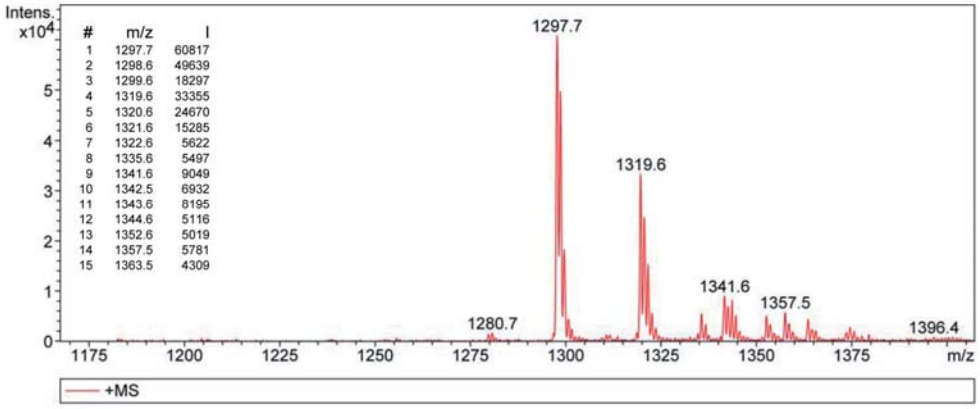


Figure 11. MS of [D-Tyr^4]Angiotensin-I ($C_{62}H_{88}DN_{17}O_{14}$, M_w = 1296.7) after purification. m/z = 1297.7 ($M + H^+$), m/z = 1319.6 ($M + Na^+$), m/z = 1341.6 ($M + 2 \times Na^+$), m/z = 1362.6 ($M + 3 \times Na^+$).

Supplemental MS Spectra for reaction 1-10.

Conversion of [Iodo-Tyr^4]Angiotensin-I to the labelled [D-Tyr^4]Angiotensin-I was calculated from MS-spectra intensities (I), product / (product + starting material). The ionization of the substrate and product peptide are estimated to be approximately equal in the ESI interface. All spectra where recorded using the same acquisition parameters as follows.

Mode	
Mass Range Mode	Std/Normal
Ion Polarity	Positive
Ion Source Type	ESI
Alternating Ion Polarity	off
Current Alternating Ion Pol	Positive
Divert Valve	to Source

Tune SPS	
Target Mass	1300 m/z
Compound Stability	90 %
Trap Drive Level	90 %
Optimize	Wide
Smart Parameter Setting	active

Tune Source	
Trap Drive	68.6
Octopole RF Amplitude	150.0 Vpp
Lens 2	-60.0 Volt
Capillary Exit	148.1 Volt
Dry Temp (Set)	300 °C
Nebulizer (Set)	30.00 psi
Dry Gas (Set)	8.00 l/min
HV Capillary	4500 V
HV End Plate Offset	-500 V

Trap	
Rolling	on
Rolling, Averages	4 cts
Scan Begin	500 m/z
Scan End	2000 m/z
Averages	8 Spectra
Max. Accu Time	200000 µs
ICC Target	20000
Charge Control	on

MS/MS Automatic	
Auto MS/MS	off

Fragmentation Options	
CutOff Selection	Default
SmartFrag	Off
Fragmentation Width	10.00 m/z
Fragmentation Time	40000 µs
Fragmentation Delay	0 µs

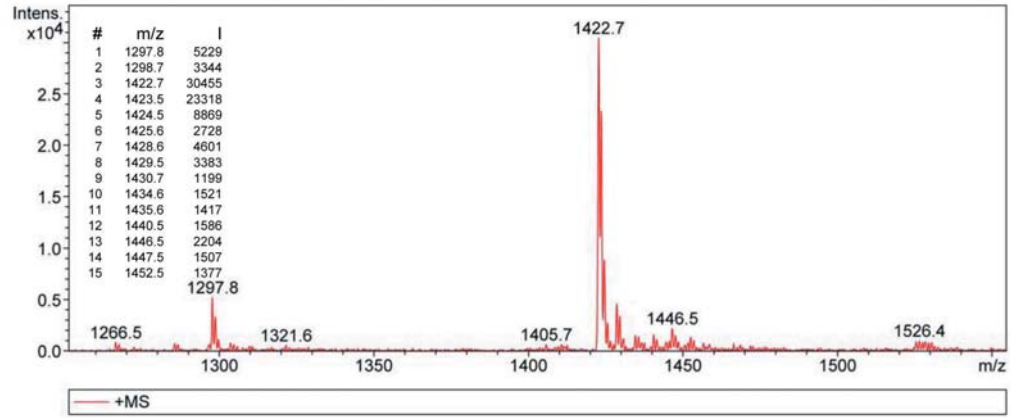


Figure 12. MS of reaction 1. [D-Tyr^4]Angiotensin-I, m/z = 1297.8 (M + H$^+$); [Iodo-Tyr^4]Angiotensin-I, m/z = 1422.7 (M + H+). Conversion: 14.7%

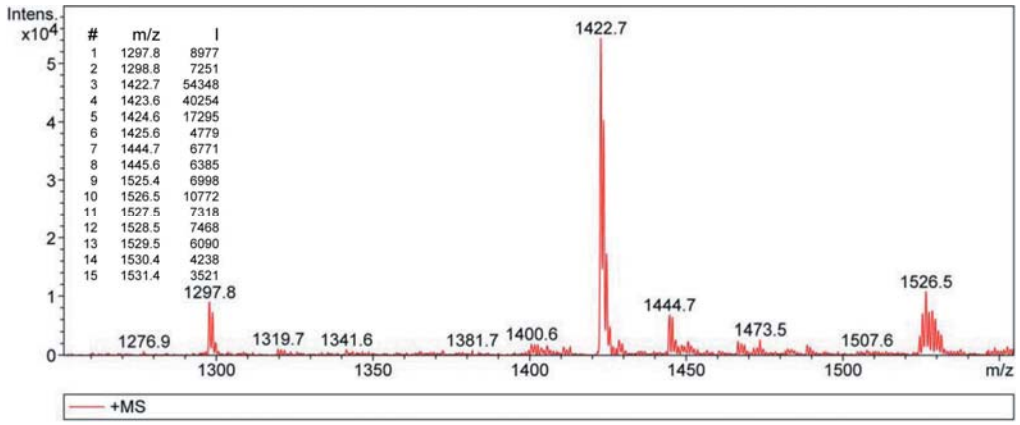


Figure 13. MS of reaction 2. [D-Tyr^4]Angiotensin-I, m/z = 1297.8 (M + H$^+$); [Iodo-Tyr^4]Angiotensin-I, m/z = 1422.7 (M + H$^+$), m/z = 1526.5 (M + Pd(0) + H$^+$). Conversion: 14.2%.

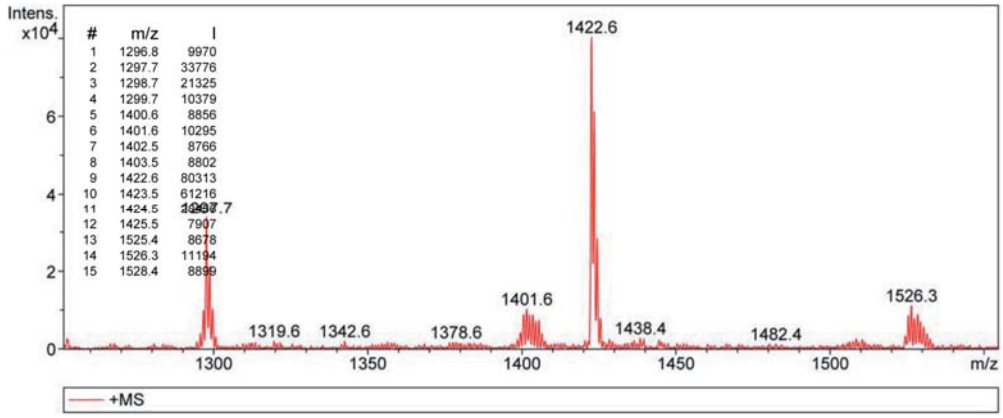


Figure 14. MS of reaction 3. [D-Tyr^4]Angiotensin-I, m/z = 1297.7 (M + H$^+$), m/z = 1401.6 (M + Pd(0) + H$^+$); [Iodo-Tyr^4]Angiotensin-I, m/z = 1422.6 (M + H$^+$), m/z = 1526.3 (M + Pd(0) + H$^+$). Conversion: 29.6%.

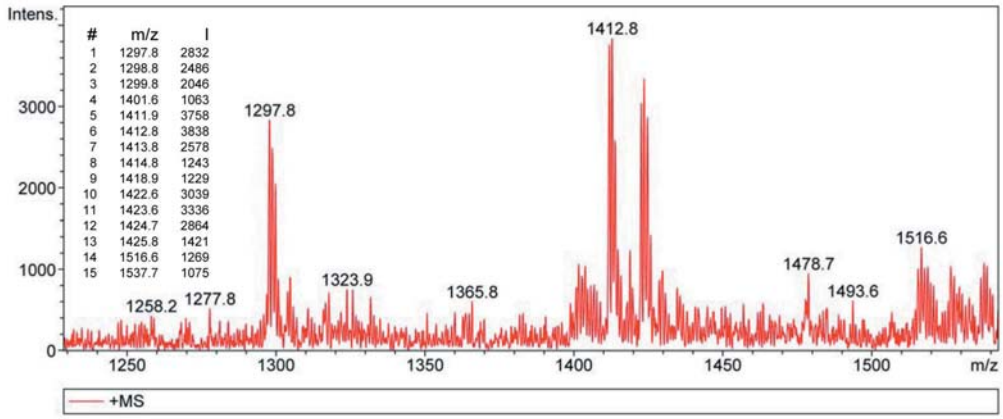


Figure 15. MS of reaction 4. [D-Tyr^4]Angiotensin-I, m/z = 1297.8 (M + H$^+$), m/z = 1401.6 (M + Pd(0) + H$^+$); [D-Tyr^4]Angiotensin-I + triethylsilanyl, m/z = 1412.8 (M + H$^+$), m/z = 1516.6 (M + Pd(0) + H$^+$); [Iodo-Tyr^4]Angiotensin-I, m/z = 1422.6 (M + H$^+$). Conversion: 68.7%.

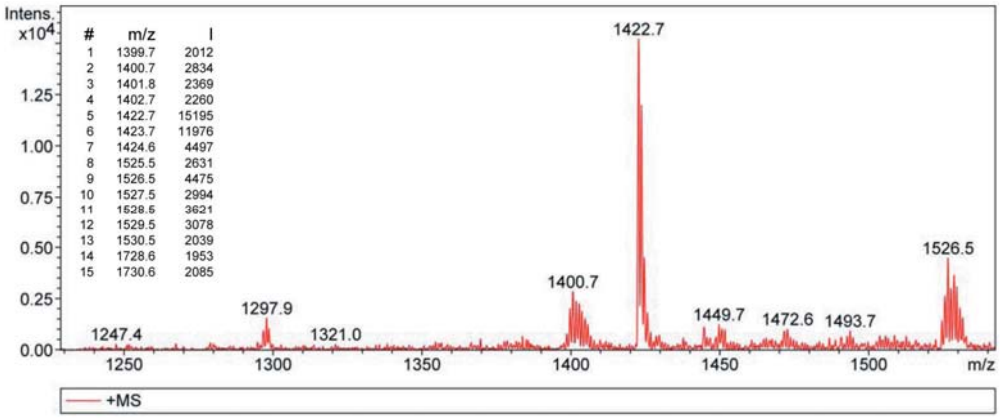


Figure 16. MS of reaction 5. [D-Tyr^4]Angiotensin-I, m/z = 1297.9 (M + H$^+$), m/z = 1400.7 (M + Pd(0) + H$^+$); [Iodo-Tyr^4]Angiotensin-I, m/z = 1422.7 (M + H$^+$), m/z = 1526.6 (M + Pd(0) + H$^+$). Conversion: 0.9%.

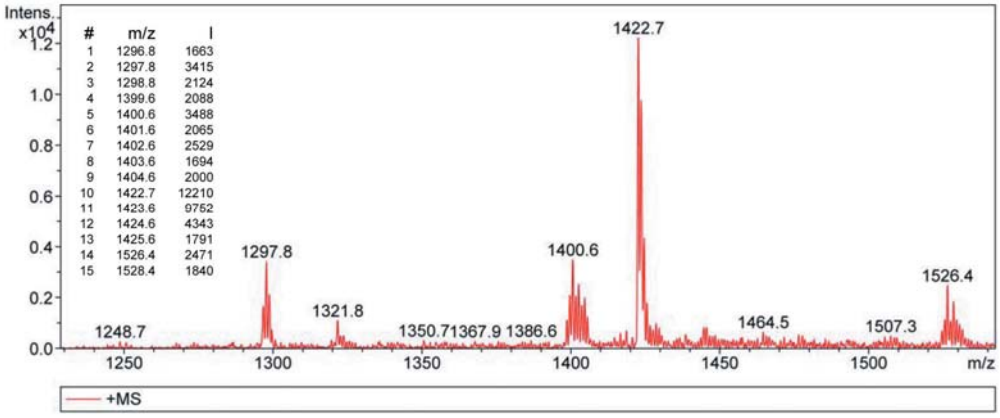


Figure 17. MS of reaction 6. [D-Tyr^4]Angiotensin-I, m/z = 1297.8 (M + H$^+$), m/z = 1400.6 (M + Pd(0) + H$^+$); [Iodo-Tyr^4]Angiotensin-I, m/z = 1422.7 (M + H$^+$), m/z = 1526.4 (M + Pd(0) + H$^+$). Conversion: 21.9%.

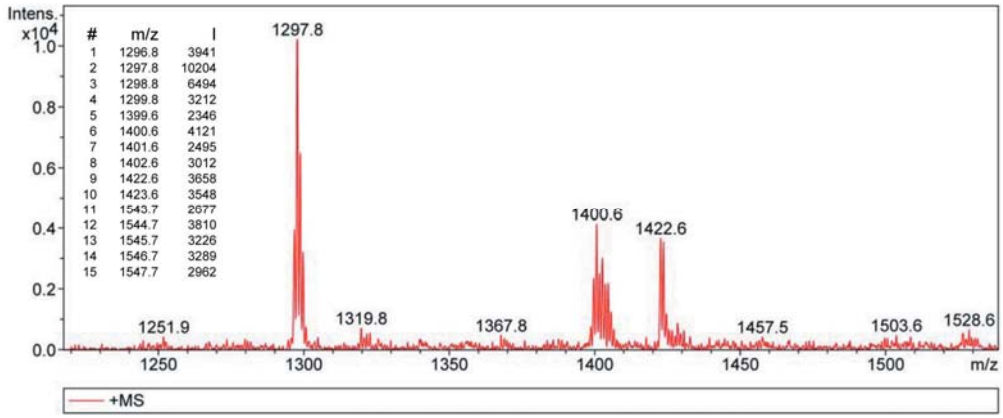


Figure 18. MS of reaction 7. [D-Tyr^4]Angiotensin-I, m/z = 1297.8 (M + H$^+$), m/z = 1400.6 (M + Pd(0) + H$^+$); [Iodo-Tyr^4]Angiotensin-I, m/z = 1422.6 (M + H$^+$). Conversion: 73.6%.

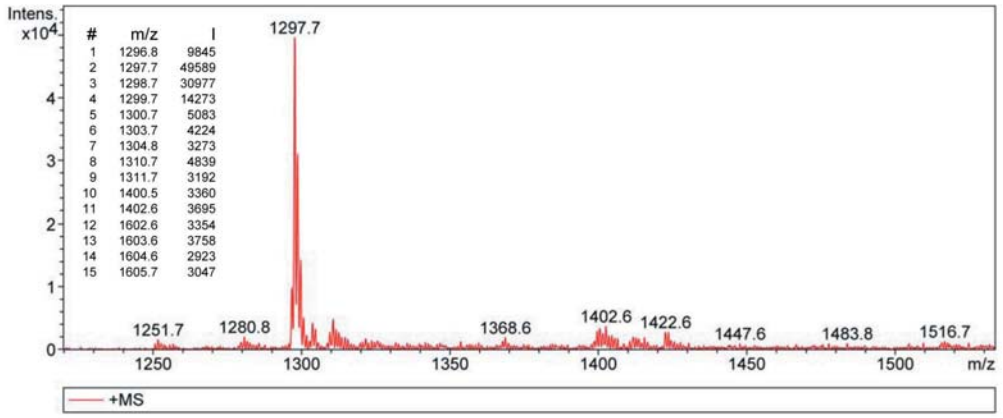


Figure 19. MS of reaction 8. [D-Tyr^4]Angiotensin-I, m/z = 1297.7 (M + H$^+$), m/z = 1402.6 (M + Pd(0) + H$^+$); [Iodo-Tyr^4]Angiotensin-I, m/z = 1422.6 (M + H$^+$). Conversion: Nearly 100%.

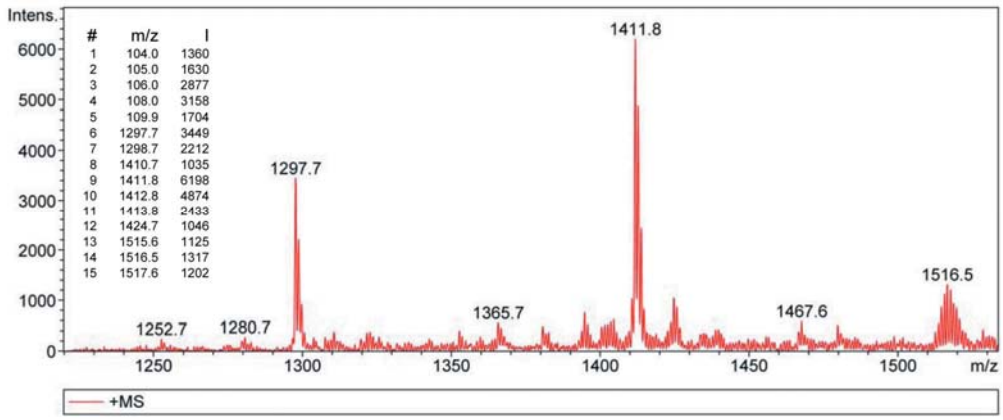


Figure 20. MS of reaction 9. [D-Tyr^4]Angiotensin-I, m/z = 1297.7 (M + H$^+$); [D-Tyr^4]-Angiotensin-I + triethylsilanyl, m/z = 1411.8 (M + H$^+$), m/z = 1516.5 (M + Pd(0) + H$^+$). Conversion: Nearly 100%.

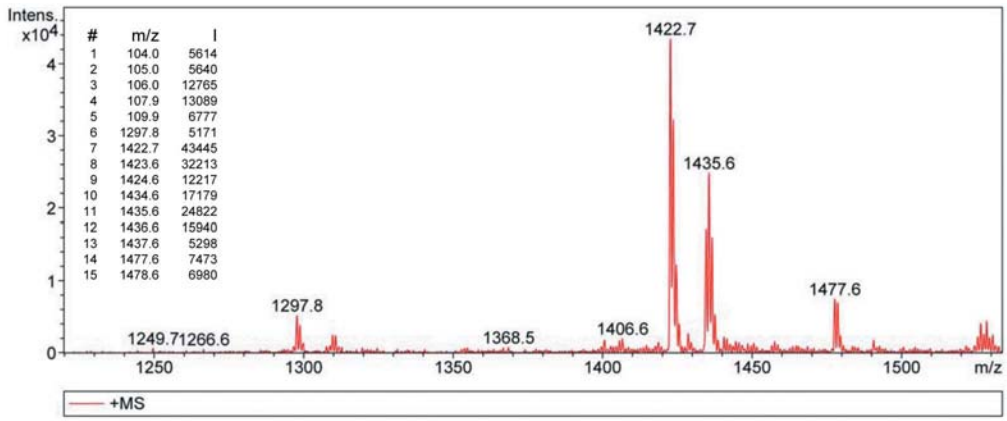


Figure 21. MS of reaction 10. [D-Tyr^4]Angiotensin-I, m/z = 1297.8 (M + H$^+$); [Iodo-Tyr^4]Angiotensin-I, m/z = 1422.7 (M + H$^+$).
Unidentified compounds, m/z = 1435.6, m/z = 1477.6.

Appendix II.

Radioiododestannyllation of Novel GHB Neuroreceptor Ligands

Preface and Background

A series of radioiodination labelling reactions of small molecules have been performed as side projects in this graduate work. One of these, the labelling of a gamma-hydroxybuturic acid (GHB) receptor ligand, was presented on the 10th International Isotope Society symposium in Chicago, 2009.

This is why a short overview of radioiodine, this elements isotopes and presentation of the very useful radioiododestannyllation (for labelling of small molecules) are presented here. The conference proceeding with chemical results of this one labelling is a representative illustration of the usefulness of this reaction. Research and biological uses of these receptor ligands are submitted for publication elsewhere.

Isotopes of iodine are very often used in pharmaceutical R&D and in clinical practices. Around 30 radioisotopes of iodine have been reported, where the only stable is iodine-127. The ease of chemistry with iodine/iodide makes radioisotopes of this element very attractive to use as tracers, not only for small molecules but also for peptides and proteins.[1]

Isotope	Decay*	$T_{½}$	Uses
Iodine-123	EC	13.2 *h*	Considered "Ideal" isotope for thyroid imaging.
Iodine-124	EC (24% β^+) Hard energy CE and γ radiation (>600 keV)	4.2 *d*	Potential PET isotope, though not used heavily in humans due to dose considerations from the hard electron/gamma emissions.
Iodine-125	**EC** **Soft energy CE and x-ray emission (max 35 keV)**	**59.4 *d***	**Used in *in-vitro* pharmacology studies. Evaluation of receptor ligand affinity.**
Iodine-127	-	Stable	-
Iodine-131	β^- Medium/hard energy CE and γ emission (max 350 keV)	8.0 *d*	Classical isotope for thyroid uptake and imaging purposes. Have been used for several decades for this purpose.

Table 3. Short list of the most frequently used radioiodine isotopes in R&D and clinical practises. *The iodine isotopes possess complex decay patterns for more details refer to the NUDAT 2.5 database[2].

One of the proven very useful methods for labelling with iodine is the radioiododestannyllation reaction. With the use of an organic oxidant, such as chloramine-T, in combination with iodide (I^-), the in-situ generation of an electrophilic iodide ($I^{\delta+}$) gives the possibility for reaction on a "nucleophilic" carbon.

1. Welch, M. J.; Redvanly, C. S., *Handbook of Radiopharmaceuticals - Radiochemistry and Applications*. John Wiley & Sons Ltd.: 2003.
2. NuDat 2.5. National Nuclear Data Center and Brookhaven National Laboratory. http://www.nndc.bnl.gov/nudat2/

Radioiododestannyllation of Novel GHB Neuroreceptor Ligands

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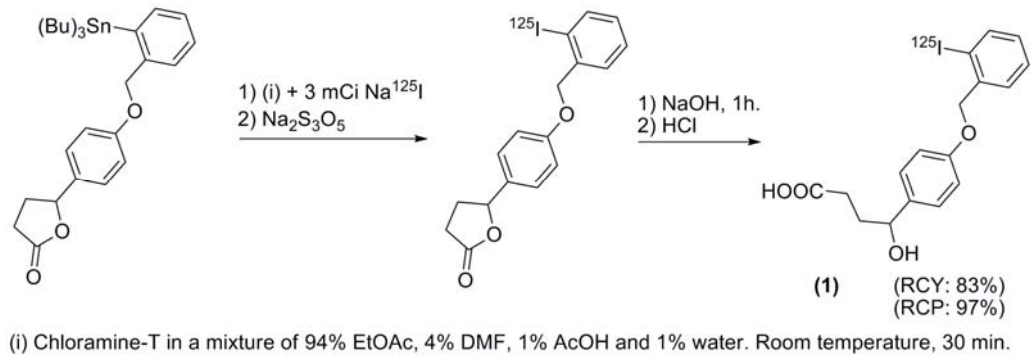
Abstract

A series of 4-substituted gamma-hydrobutyric acid analogues have been synthesized and subjected to a specific GHB binding assay[1, 2]. Within this series some halogenated analogues showed very good displacement of a specific GHB receptor binder. Here we present how we have used the synthesis of two I-125 labelled GHB neuroligands by radioiododestannylation. The I-125 labelled compounds **1** and **2*** were readily prepared (Scheme 1) and are currently undergoing pharmacological evaluation in the hope for elucidation of the GHB binding sites and GABA neuroreceptors. The tributyl-stannate precursor in its lactone form was reacted with electrophilic 125-iodine (generated from carrier-free $Na^{125}I$ in presence of chloramine-T). Efficient labelling was achieved within 30 minutes and subsequent deprotection achieved by lactone hydrolysis. RP HPLC purification and reformulation using SEP PAK C18 cartridges, rinsing with purified water, elution with ethanol and finale dilution with purified water gave the labelled compounds **1** and **2*** in excellent radiochemical yields (83% and 88% respectively).



Scheme 1. Electrophilic radioiodo-destannyllation followed by lactone hydrolysis.

Short Introduction

Gamma-hydroxy-buturic acid (GHB) is a small neuroreceptor ligand with an affinity for the gamma-aminobutoric acid (GABA) receptors. It is a metabolite of GABA and has been found in micromolar concentrations in the mammarial brain[3]. The compound is listed as an illicit drug, because of its drug-abuse potential (street name: Fantasy) and examples of uses as a date rape drug[4]. The pharmacological effects of the compound make it useful for the treatment of cataplexy, a symptom associated with the excessive daytime sleeping (Narcolepsy) condition[5, 6]. GHB is also, therefore, a registered pharmaceutical drug called Xyrem® formulated as the sodium oxybate[7]. Because of the public concern about the misuse of GHB along with the pharmaceutical use, investigation of the pharmacodynamics of GHB is required.

Radioiododestannyllation has been proven to be a widely applicable method for labelling organic molecules with radioiodine[8]. Electrophilic iodine can be created (as $I^{\delta+}$–$Cl^{\delta-}$) *insitu* by the reaction

of NaI with the oxidant Chloramine-T[9]. This has proven to be an efficient method for radiolabelling with radioiodine. In this example we have used a palladium coupling reaction for the synthesis of the organostannate using hexa-*n*-butyl-ditin and tetrakistriphenylphosphine-palladium.

Proceeding

(1) 4-hydroxy-4-[4-(2-iodobenzyloxy)phenyl]butanoic acid. Chloramine-T trihydrate (40.8 mg, 145 μmole) was suspended in a mixture of EtOAc (4.75 mL), DMF (0.25 mL), acetic acid (50 μL) and a small amount of water were added (50 μL) to dissolve the compound. This reagent solution (1000 μL) was added to the tin precursor (1.19 mg, 2.1 μmole) in a small HPLC vial, the vial was capped and shaken to dissolve the compounds. The I-125 NaI (30 μl, 3 mCi) was added to the vial and which was reclosed quickly after. The vial was shaken and left to react for 30 min.

Sodium metabisulfite (9.70 mg) in water (350 μL) was added to quench excess Chloramine-T and the reaction mixture was washed with purified water (3 x 300 μL). The organic layer was evaporated to dryness under an argon flow.

Subsequently, the reaction mixture was dissolved in acetonitrile (500 μL) and NaOH (20 μL, 4M) the latter to hydrolyse the lactone. The sample was shaken and left night over at 5^{o}C, then it was neutralized with HCl (80 μL, 1M) and immediately subjected to HPLC for purification.

The desired product **1** was collected at 24.7 minutes, then analysed to have a radiochemical purity of 97% and a total activity of 2.48 mCi (83%). HPLC Conditions: Buffer A (4.9% MeCN, 95% Water, 0.1% TFA), Buffer B (99.9% MeCN, 0.1% TFA). Gradient: 0 min. to 15 min. (30% B); 15 min. to 35

min. (gradient to 100% B); 35 min. to 45 min. (100% B); 45 min. to 46 min. (gradient to 30% B).

The product was diluted with five times of ultra purified water and loaded onto a pre-activated C-18 Sep-PAK (160 mg). It was then washed with ultra purified water (5 mL), eluted with EtOH (5 mL) and subsequently diluted to a concentration of approximately 5 MBq/ml with a dilute NaOH solution (0.001M).

The radiochemical analysis (Figure 1) was performed on the same HPLC system as the preparation, with a Rarroll Ramsey S105 radio detector equipped with a silicon diode.

(2)* Fotoligand. The fotoligand with similar structure as **1** was labeled with the same procedure described above. Since it was a light sensitive compound all experiments were performed under minimal light conditions. The desired iodinated product **2** was collected at 26.7 minutes, then analysed to have a radiochemical purity of 100% and a total activity of 1.75 mCi (88%).

* The structure of **2** is yet to be revealed as investigation of the compound is ongoing in the laboratory.

The radiochemical purity and analysis (Figure 2) was determined by the same method as with compound **1**.

Figure 1. HPLC Radiogram for RCP of **1.**

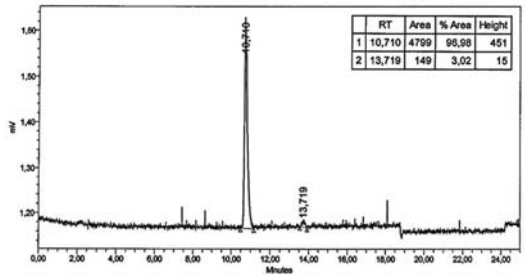
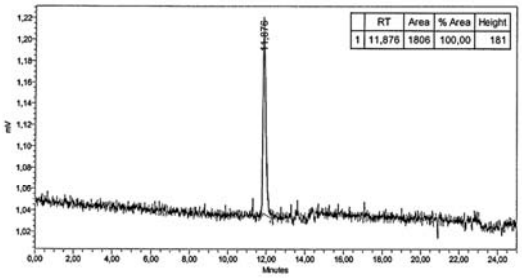


Figure 2. HPLC Radiogram for RCP of **2.**



Material and Methods

Chemicals were used as arrived from the supplier. Carrierfree Sodium[125-I]Iodine was purchased from Perkin Elmer with a radioactive conventration of 10 mCi/100 µL.

Acknowledgements

The work on design and synthesis of GHB ligands has been supported by The Danish Medical Research Council, Beckett-fonden and "Carl og Ellen Hertz Legat for Dansk Natur- og Lægevidenskab", all performed at the Institute of Medicinal Chemistry, University of Copenhagen, Denmark. Researchers of who are gratefully acknowledged as a part of this are Hans Bräuner-Osborne, DSc, Rasmus P. Clausen, PhD, Bente Frølund, PhD (Inst. of Med. Chem., Uni. of Copenhagen) and Gitte Moos Knudsen, DMSc (Neurobiology Research Unit, Rigshospitalet). Radiolabelling experiments where performed at the Hevesy Laboratory, Radiation Research Division, Risø-DTU, The Technical University of Denmark, who are gratefully thanked for the supply of radioisotopes and instruments necessary for the labelling.

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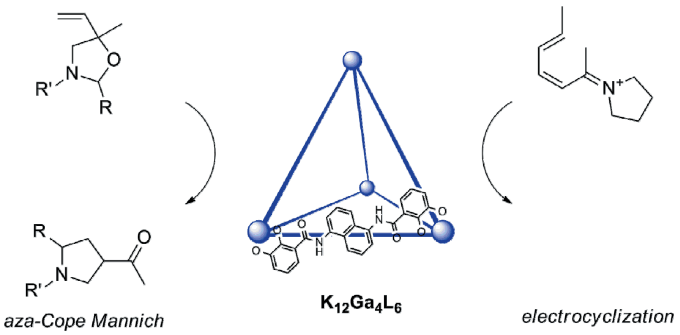
Part III.

Investigation of Guest-Host Mediated Electrocyclization & Sigmatropic Rearrangement Reactions



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Front page: The graphics on the front illustrates the investigated reactions with the supramolecular assembly.

Preface to Part III.

This part concludes the investigation of small molecule transformations and catalysis with the supramolecular self-assembled cluster $[Ga_4L_6]^{-12}$ from this point on represented by (**1**). The focus of the research was on cationic reactions and has taken advantages of earlier investigations of the cluster. In this part two types of reactions are described; cationic 6π electrocyclizations of iminium/ammonium substrates and proton-mediated cationic sigmatropic rearrangements (aza-Cope Mannich) in basic solutions.

The research presented in this part was performed in the laboratories of Prof. Robert G. Bergman, Department of Chemistry, University of California, Berkeley. An eight month period of the total three year phd program was spent in his laboratories. During this research visit, studies were conducted on organic reactions mediated by a rationally-designed spontaneously-assembling supramolecular cluster that was developed in the laboratory of Prof. Kenneth Raymond, Dept. of Chem., UC Berkeley.

IN SUMMARY THIS PART CONSISTS OF;

1) A short introductory/background section to the field of "*supramolecular chemistry"* and *"the cluster"*,

2) A section which is divided into three sections describing the two main reaction types in focus, "*6π electrocyclization*" and "*sigmatropic rearrangement*". Before each of these sections, a "*short background"* and "*introduction to the research ideas*" is presented and thus left out of the main introduction chapter.

3) And last "*the experimental section*".

The research I have conducted in the Bergman group do not have any direct association with my radiochemistry phd project performed at the Risø National Laboratory, DTU, but shall be seen as an independent part of my graduate studies. The Bergman group focuses on a broad range of research subjects within the area of inorganic, organometallic and physical organic chemistry. Specifically, in collaboration with Prof. Kenneth Raymond, the exploration of organic chemical transformations and catalysis using supramolecular assemblies is an area of significant interest in the Bergman group.

The initial idea for the project was to utilize host-guest methodology for the mediation of 6π-electrocyclization reactions with the $[Ga_4L_6]^{-12}$ supramolecular cluster (**1**). After spending time on this project, the focus of research eventually turned to literature known sigmatropic rearrangements. In my hands, the latter project proved to be more successful.

The stay at UC Berkeley has become an experience of my lifetime. It has given me a more thorough understanding of general synthetic and organometallic chemistry and

the approach to scientific questions. Furthermore, the opportunity for an international adventure and to work with different scientific disciplines has in every sense broadened my basic view of how research is to-be conducted. In my research visit, I have worked with supramolecular chemistry, a branch of chemical sciences that was an all new experience for me. It is a broad chemical branch that I think will become very prominent in the future. It is already used in a small degree in radiochemistry (e.g. cryptants for $K^{18}F$ labeling reactions and as chelators for radioisotopes) - maybe it will eventually find a broader application in this, my possible future, field.

Acknowledgements

I am thankful for my experiences and to have this opportunity to be a visiting scholar at UC Berkeley. This was first of all possible because my graduate program defines this option of an independent research visit abroad, for this I am much appreciative.

My special gratitude goes to:

Prof. Robert G. Bergman, Department of Chemistry, UC Berkeley, for the supply of laboratory space and research equipment. Especially I would like to express my gratitude for the research meetings – the "Bob-talks" – and the group meetings; these were always interesting, inspiring and often led to in depth academic discussions. Experiences that I believe have broadened my view on how to work and interpret chemical and scientific work in general. I would also like to express my gratitude for the opportunity to work on the supramolecular catalysis project in collaboration with **Prof. Kenneth Raymond**, Department of Chemistry, UCB.

Anneke Runtipalit, administratice assistant, Department of Chemistry, UC Berkeley. Special thanks to Anneke for being very helpful with taking care of the administrative necessaries and in general making my visit a pleasant time.

Lee Bishop and **Courtney Hastings**, 5th year graduate students whom I would like to thank for being my personal tutors and supervisors in the Bergman Group. They have contributed with lots of inspiration and ideas. I would like to acknowledge both for giving me their time, great chemistry discussions and collaboration.

Dr. Mark Crimmin, post. doc. at the Bergman group for many great chemical discussions, support thoughout my stay and proof reading of this research report.

Background: “Chemistry Beyond the Molecule”

Definition of Supramolecular and Host-Guest Chemistry.

Supramolecular chemistry is the definition of a sub-group of the chemical sciences that was defined by one its leading proponents, Dr. Jean-Marie Lehn. He received the Nobel Prize for his work in the area in 1987,[1, 2] "for….development and use of molecules with structure-specific interactions of high selectivity". In the late sixties/early seventies, Lehn and co-workers achieved the synthesis of cage-like molecules, biaza-polyoxo-macrobicycles (cryptants), and showed that these molecules have the ability to molecular recognize Group I cations[3]. Originally the term supramolecular chemistry was used to define the chemistry of non-covalent interaction between a host molecule and a guest as depicted in figure 1.

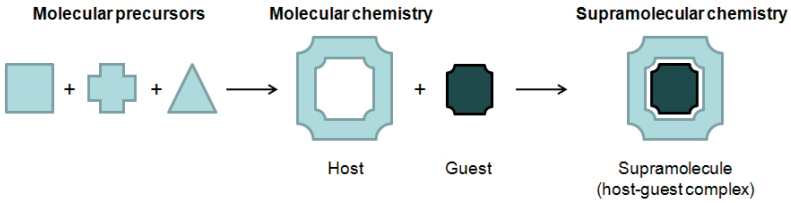


Figure 1. The basic concept of molecular host – and non-covalent recognition of guests with high selectivity.

Whilst defining an area of chemical research, the term “supramolecular chemistry” incorporates long-standing ideas that encompass a number of chemical fields. Non-covalent complexes and non-covalent interactions upon the molecular level are found widely in most of the chemical sciences and, perhaps more importantly, in the systems that drive life, such as enzymes (a supramolecular catalysis process) and neuroreceptors (a supramolecular complex respons). The field of supramolecular chemistry has progressed towards an informational science; underpinned by the understanding of chemical self-organization processes and the use of this knowledge to gain control of small molecule transformations through host-guest recognition processes.[4] The field has gained enormous attention in the last couple of decades and numerous examples of chemical systems, either designed or accidently created, that would belong under this definition have been reported.

Supramolecular chemistry, in its simplest form, involves the non-covalent but specific binding of a guest molecule or element to a molecular host. Binding of the host to the guest forms a host-guest complex. Important questions arise as to why selective and strong binding occur. The host molecule is defined as a molecular structure with binding sites with the capacity to bind small molecules through hydrogen-bond donating or accepting groups, ion-pair (electrostatic), van-der-waals, π-acid or π-base interactions or metal to ligand binding. In general there must be an entropic favor for the guest to bind strongly to the host structure. Donald Cram, the fellow supramolecular chemistry Nobel Laureate of Lehn, explained the host-guest concept in a series of papers[5].

Supramolecular Catalysis

Within the world of biology/biochemistry there are numerous examples of extraordinary molecular transformations performed by enzymes – reactions that proceed with remarkable efficiency and selectivity. One could argue that beginning from the origin of life (spontaneous generation and self-replication) molecular complexity has increased. Enzymes found in nature have a very high specificity and in most cases suffer from only limited product inhibition. The enzymes or enzyme mimic (in the case of this part; the supramolecular assembly) are able to increase the rate of reactions by orders of magnitude, if compared to the uncatalyzed reaction. An increase in reactivity initiated through chemical recognition of a substrate, the guest molecule[6]. In figure 2, the very basic transformation of substrate to product is represented schematically. The fundamental ideas behind this model were proposed by Pauling more than 60 years ago; the initial molecular recognition and stabilization of the transition state of the reaction.[7] Enzymes are dynamic macromolecules with molecular weights that in general exceed 10 kDa. They are made of polypeptides that fold into unique secondary (alpha helix and beta sheet), tertiary and quaternary structures depending on the amino acid sequence, chemical environment and the intra- and inter- molecular interactions of the sub-units.

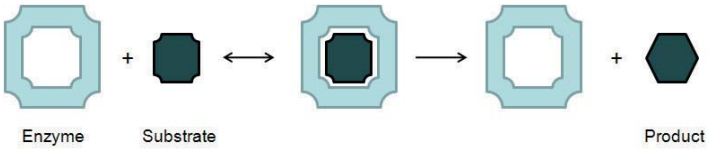


Figure 2. Basic schematic of enzyme (supramolecular catalysis) process.

The biological systems have provided many of the ideas and are the inspiration behind the development of supramolecular chemistry. Supramolecular systems have been designed to mimic the structure and function of much more complex biological processes,[8, 9] and it has been a dream for chemists to create artificial enzymes for a long time. Indeed, some supramolecular hosts that have been synthesized within the laboratory to possess catalytic activity.

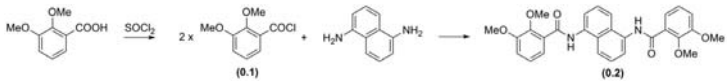
From this point onwards this part will focus on the supramolecular assembly and the experiments that have performed with this Host.

Introduction to and Synthesis of The Assembly.

The supramolecular spontaneously self-assembled cluster ($K_{12}Ga_4L_6$ where L = N,N'-bis(2,3-dihydroxy-benzoyl)-1,5-diaminonaphtalene) or compound (**1**), investigated in this work will be, in this part, be referred to as: (the) cluster, assembly, host, catalyst or nano-vessel. Structure and formation of (**1**) are shown on next page. The ligand span are illustrated by blue lines and the metal centers by the red spheres.

The assembly (**1**), was first synthesized in 1998 in the group of Prof. Kenneth Raymond, UC Berkeley.[10] This supramolecular structure is a tetrahedral assembly, based on six bis-bidentate ligands which span the tetrahedron edges and four octahedral gallium metal centers (M_4L_6 stoichiometry) which sit at the vertices. The structure of this assembly was designed based on the symmetry of the ligand to metal centers, and was correctly predicted using molecular modeling prior to its synthesis.[11] The assembly contains a large internal cavity that is capable of accommodating guests of various sizes.

The synthesis of the supramolecular cluster is uncomplicated.[10, 12] Synthesis of the ligand (H_4L, **0.3**) is outlined in scheme 1 and 2. The first step is a diacylation of 1,5-diaminonaphtalene for the formation of (**0.2**).



Scheme 1. Diacylation of 1,5-diaminonaphtalene.

The second step is a global demethylation of the four aryl methyl ether groups for formation of (**0.3**). This demethylation is performed over a period of 5 days at ambient temperature in boron tribromide (scheme 2).

Scheme 2. Tetrademethylation to form H_4L.

The assembly is then formed by the reaction of 6 : 4 : 12 mixture of (**0.3**), $Ga(acac)_3$ and potassium hydroxide in MeOH - A reaction that proceeds via a stepwise deprotonation of the ligand, followed by a complexation to gallium to form the self-assemblied cluster. The cluster (**1**) can be precipitated out of the reaction by reducing the solvent volume and subsequent slow addition of Et_2O or acetone. The reaction and formation of (**1**) is outlined in scheme 3.

Scheme 3. Formation of $[K_{12}Ga_4L_6]$ or compound (**1**).

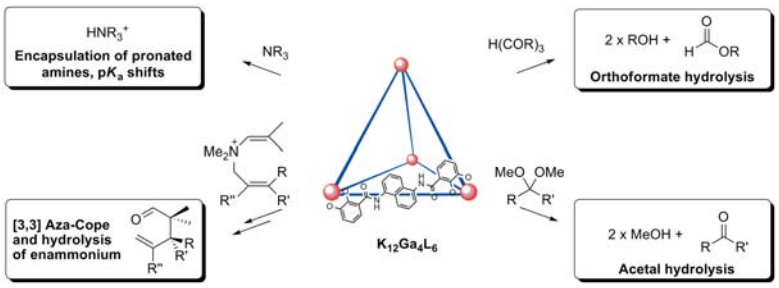
Even though there is no stereogenic carbon present in the ligand the self-assembly is chiral as synthesized. In the synthesis of the molecular structure (**1**) four stereogenic centers are formed around the tris-bidentate metal centers. This produces the two homochiral ΔΔΔΔ and ΛΛΛΛ configured products.[10, 13] As isolated the cluster is

racemic, although further experiments have shown that it may be resolved using the chiral cation S-nicotinium as the guest.[14]

Short Introduction to Reactivity of The Assembly.

The highly anionic, water soluble assembly (**1**) encapsulates a variety of both neutral and cationic molecules, and can also catalyze molecular transformations within the cavity. The hydrophobic cavity of the assembly strongly binds appropriately sized cationic guests, including alkylammonium salts and organometallic complexes.[15, 16] Interestingly the cluster was found to recognize and stabilize iminium ions (formed by the addition of secondary amines to ketones) in water, as well as making amines stronger bases by protonation and encapsulation.[17] The internal solvent accessible volume of the assembly have been estimated to be 250 – 430 Å^3, depending upon the size of the guest molecule. The nature of the amide bonds in the ligands, gives it a degree of flexibility, allowing overall conformity change of the structure and provides a dynamic encapsulation environment that may respond to the size of the guest.[18] In aqueous solution, the major driving force for guest encapsulation has been proposed to be solvent reorganization.[19, 20]

The assembly can also serve as a nano-sized reaction vessel, and can catalyze reactions in aqueous media. One of the early examples reported was the aza-Cope rearrangement of enammonium substrates,[21-23] followed by hydrolysis of the iminium product to form aldehydes. This reaction is accelerated by binding the substrate molecule in a reactive conformation within the confinement of the interior cavity of (**1**), lowering the entropic barrier for the reaction. Another remarkable property of this cluster is that it enables proton-mediated reactions under basic conditions by increasing the basicity of bound guests.[24] It was shown to catalyze acetal deprotection to form carbonyls[25, 26] and catalyze the hydrolysis of orthoformates[27]. Very interestingly, the hydrolysis was shown to follow the Michaelis-Menten model of enzyme kinetics[28] – inhibition with a competitive inhibitor (Pr_4N^+) in the orthoformate hydrolysis experiments confirmed enzyme-like reactivity (figure 3). A short selection of the known reactivities of the assembly is out-lined in scheme 4.



Scheme 4. Overview of selected Encapsulations and Catalytic Reactivities of $[Ga_4L_6]^{-12}$.

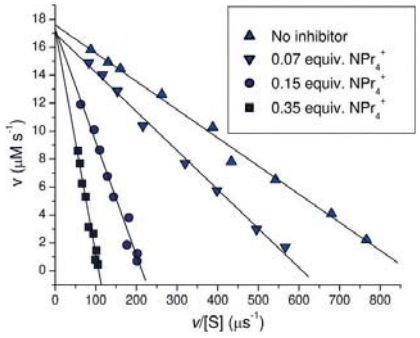
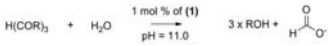


Figure 3. Eadie-Hofstee plot showing the inhibition of the catalytic hydrolysis of $HC(OEt_3)_3$ with NPr_4^+ as the competitive inhibitor in H_2O, pH=11, 50°C and 4.0 mM of (**1**).



Plot shows the decrease in initial velocity of the hydrolysis rate with increased equivalents of the inhibitor.

The limitations and scope of cluster encapsulation and reactivity are based on the size and selectivity towards substrates. The size of substrate is important; too large substrates do not encapsulate and will thus not react. To overcome this problem, the Raymond group, have synthesized clusters with extended ligands based on a pyrene bridge.[29]

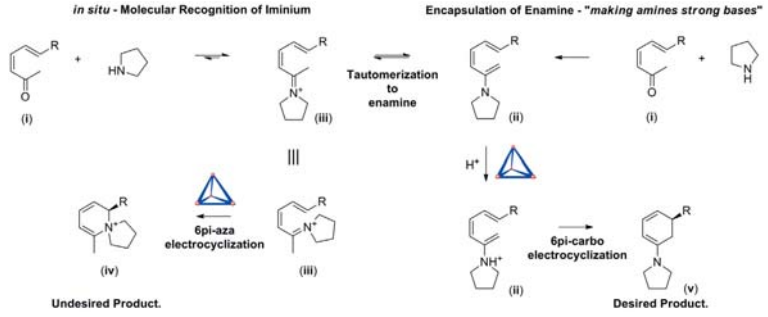
Investigated Reactivity with The Assembly

In the work presented here, studies have been undertaken to investigate and expand the scope of, the use of (**1**) as a catalyst for transformation of appropriately-sized cationic substrates (or intermediates) *via* host-guest encapsulation. This section has been divided into further two sub-categories based upon the reactiontypes investigated – "*6π electrocyclizations*" and "*sigmatropic rearrangement*s". For the substrates within this series that did not encapsulate or react in the desired way, only brief discussion of results are mentioned, whereas the more promising substrates are discussed in more detail.

6π electrocyclizations

Short background, Research Ideas and Results.

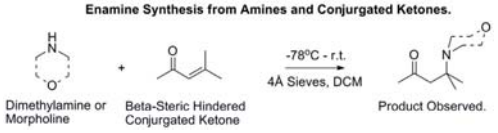
In 2008, Trauner, Bergman and coworkers published a paper about the Lewis acid catalyzed 6π-electrocyclization reaction.[30] Within this study, DFT (density functional theory) calculations suggested that electron-withdrawing groups in the 2-position of a hexatriene system would decrease the energy barrier of the transition state for the electrocyclization reaction. These calculations were supported by experiments which led to the use of catalytic amount of the Lewis acid Me_2AlCl that would coordinate to an ester substituent, increasing its electron withdrawing properties thus decreasing the transition state energy barrier and increasing the rate of reaction. Other DFT studies upon hexatrienes suggests that by altering the location and nature of the substituents, this electrocyclization reaction can proceed at much lower temperature than of an unsubstituted hexatriene.[31] The idea to facilitate this reaction type with the cluster was to-be investigated as represented in scheme 5.



Scheme 5. Proposed 6π-electrocyclization reaction mediated by the cluster.

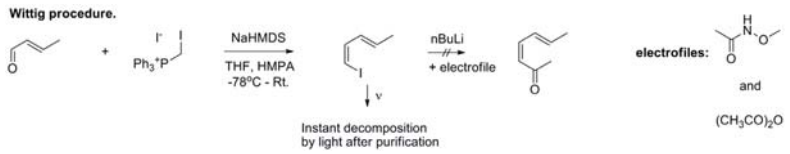
This idea could be approached in two different ways which would also take advantage of earlier work with the assembly. First approach was to synthesize a trieneamine (**ii**) and use the assembly's ability to encapsulate protonated amines hoping that the protonated trieneamine would undergo electrocyclization within the cluster; the "*encapsulation of enamine*" pathway of scheme 5. For this pathway to succeed, the challenge of synthesizing a conjugated enamine had to be overcome.

Few experiments with available model compounds rapidly concluded that it was not possible under the reaction conditions investigated (addition of amines to α,β-unsaturated ketones) and the only observed, and isolable reaction products were those formed by Michael-addition of the nucleophilic amine to the Michael acceptor, scheme 6. These experiments suggested that avoiding Michael addition chemistry would be a big challenge. As result we changed the focus to the other possible pathway and instead use the cluster's ability of "*molecular recognition of iminium*" (**iii**) as a possible route towards catalyzing/facilitating 6π-electrocyclizations. Followed by the encapsulation of an iminium ion, an *in-situ* iminium-enamine tautomerization and subsequent electrocyclization would yield the desired reaction products (**v**). For investigation this reactivity the central substrate to be synthesized was the dieneketone (**2.3**) or (**i**).



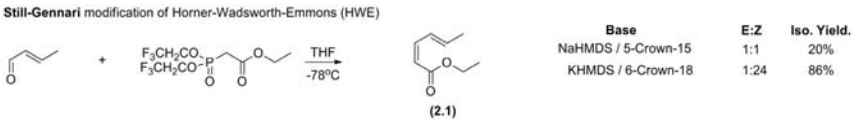
Scheme 6. Attempt for enamine synthesis of sterically hindered α,β unsaturated ketone and amine showed Michael-Addition (beta attack) and no enamine.

Attempts to synthesize the dieneketone (**2.3**) were made *via* Wittig chemistry depicted in Scheme 7. Although it was possible to synthesize and isolate the vinyl-iodide, the essential purification of this compound proved to be difficult – limiting the utility of this approach. The first step in this proposed synthesis pathway was initial a standard Wittig reaction followed by halogen-metal exchange and reaction of the corresponding "organolithium" with an electrophile. The vinyl-iodide proved to be an extremely fragile, temperature- and light-sensitive compound. Even though a significant amount of time was used to investigate this pathway, the synthesis of the desired unsaturated substrate via this pathway turned out to be unsuccessful in my hands.



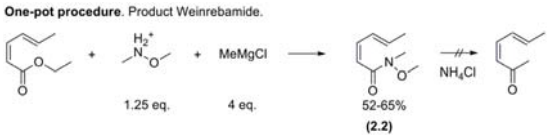
Scheme 7. Unsuccessful reaction pathway for the synthesis of (**2.3**).

Nevertheless, it was found possible to synthesize the diene-ester (**2.1**) with use of the Still-Gennari modification of the HWE (Horner-Wadsworth-Emmons) reaction. The reaction proceeded in very good yield and a high *E:Z* ratio when using the original procedure (scheme 8).[32]



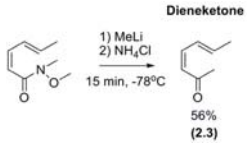
Scheme 8. Synthesis of the diene-ester (**2.1**) with the Still-Gennari modification of HWE.

The next step was the conversion of the ester (**2.1**) to the methyl-diene ketone (**2.3**). A literature procedure suggested this could be performed in an one-pot procedure with the use 1.25 eq. O,N-dimethylamino-alcohol and 4 equiv. of methyl Grignard (Scheme 9)[33]. Whilst this reaction would produce the Weinreb amide (**2.2**) *in-situ*, the extra equivalent of MeMgCl should undergo nucleophilic addition to the amide intermediate. A subsequent acidic quench would then produce (**2.3**). Despite this expectation, the reaction did not proceed to completion and stopped cleanly with the Weinreb amide (**2.2**). This product was isolated in an intermediate yield (in two attempts) and was used to create (**2.3**).



Scheme 9. Attempted one-pot procedure from ester to methyl ketone.

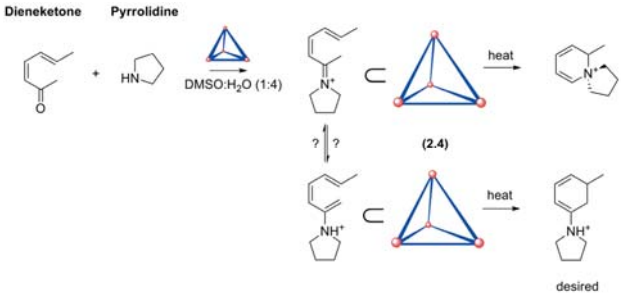
The Weinreb amide (**2.2**) was converted to the final ketone (**2.3**) by addition of one eq. methyl lithium followed by acid hydrolysis of the intermediate. The product was achieved in an intermediate yield, as is shown in scheme 10.



Scheme 10. Addition of MeLi to Weinreb amide followed by acid hydrolysis of intermediate to give (**2.3**).

With these substrates in hand, compounds (**2.2**) and (**2.3**) were investigated for reactivity with the assembly (**1**) to determine if they would form the desired iminium or enamine. The neutral substrates alone did not show any reactivity although possible encapsulation of (**2.2**) and (**2.3**) was observed by ^{1}H NMR spectroscopy.

Encapsulation of what was assumed to be the iminium from the diene ketone and amine, however, was observed and ^{1}H NMR. The general approach is shown on scheme 11 and the ^{1}H NMR followed over time shown on figure 4.



Scheme 11. Encapsulation of iminium with (**2.3**) and pyrrolidine and proposed reactivity within the cluster cavity.

As it appears from the ^{1}H NMR data a clear initial encapsulation of substrates was observed. After heating the reaction solution, the fine and defined ^{1}H NMR resonances of the encapsulated material become very complex and the resonances apparent in the high-field region of the NMR convert into many new peaks. This was a promising result and suggests reactivity within the cavity of the cluster. The product of this reaction has a higher binding constant than the substrate (determined by integration of encapsulated peaks *versus* aromatic cluster peaks) which suggests a stoichiometric reactivity with no catalytic turnover.

A standard experiment was performed with 20% cluster/catalyst loading for elucidation of catalytic activity. The product(s) of these experiments were ejected from the cluster with a strong-binding guest (Et_4N^+) and attempts were made to extract the organic product(s) into an organic solvent.

The extraction and analysis of the product(s) was in all cases unsuccessful and therefore it was not possible to identify the product(s) formed. This was unfortunate but it also suggested that it was not the desired product formed as this is expected to be soluble in organic media, but this remains unclear. The undesired aza-electrocyclization ammonium-product (scheme 11 above), which could be a proposed product, is possibly not soluble in organic solvents.

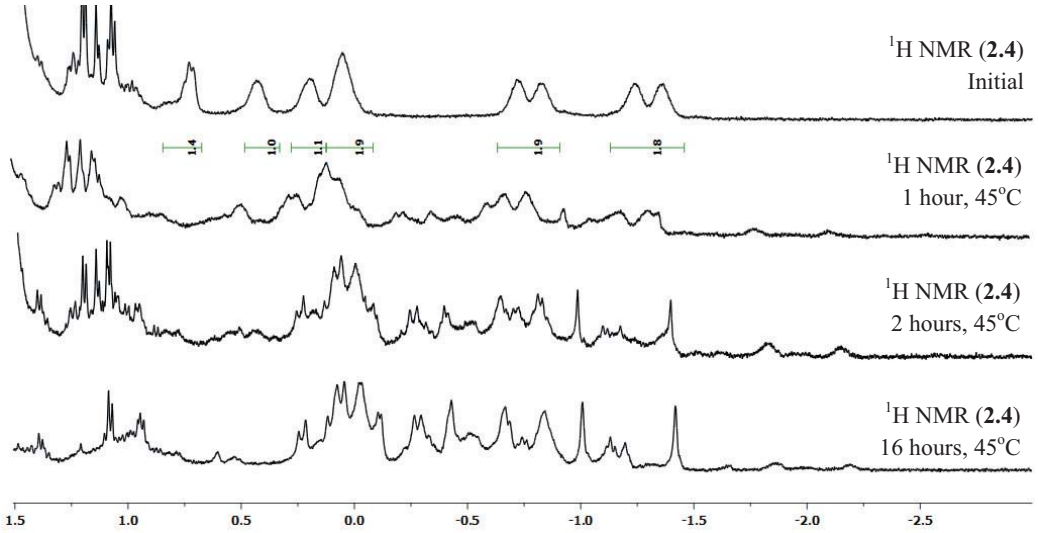
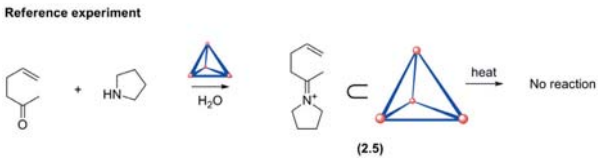


Figure 4. Illustration of the NMR spectra from experiment (**2.4**) showing the upfield/encapsulated area. The iminium ion formed showed a high binding efficiency inside the assembly, the suspected pyrrolidine sp^3 carbon hydrogen atomes integrates approximately 1H per hydrogen. After heating the NMR tube (45°C for 3 hours) the encapsulated peaks gives a more complex pattern and shows clear reactivity.

A control reaction was performed for determination of the importance of a conjugated diene π system in this electrocyclization reaction. A ketone (5-hexene-2-one) with no conjugation was reacted with pyrrolidine (**2.5**), scheme 11, and followed by ^{1}H NMR over time, depicted in figure 5. This experiment showed in contrast to (**2.4**), no reactivity over time but also good encapsulation of the iminium ion.



Scheme 11. Encapsulation of the iminium of 5 eq. non-conjugated ketone and 5 eq. pyrrolidine (**2.5**) as a reference experiment.

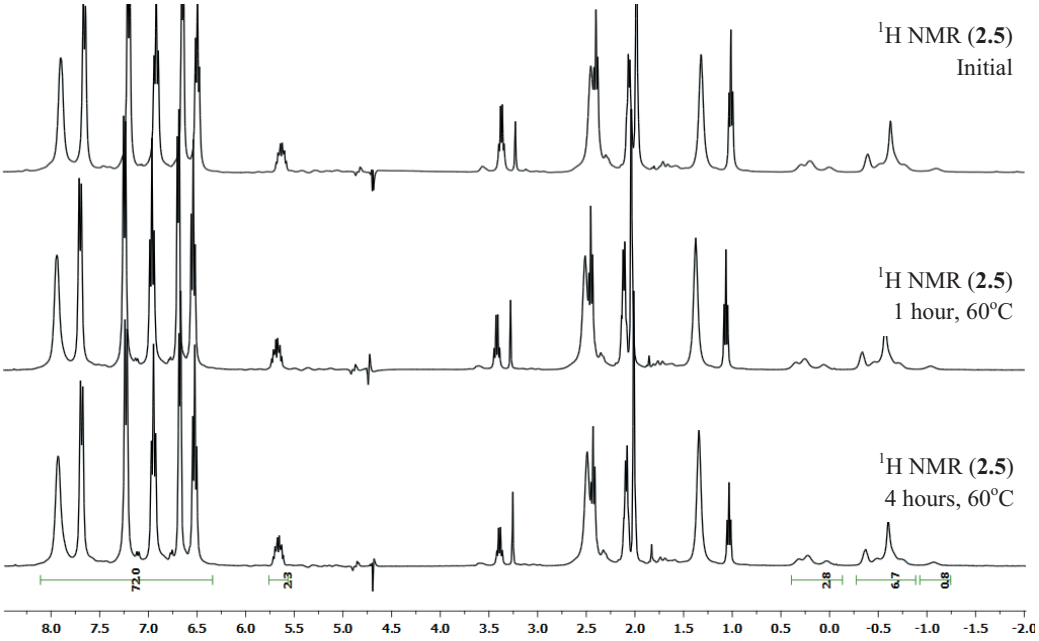


Figure 5. ^{1}H NMR of reference experiment (**2.5**) followed over 4 hours. Encapsulated area from 0.5 to -1.5 ppm, shows a clear encapsulation with ~ 11H integration which correlates to a binding efficiency of above 70% (15H on sp^3 C).

Part III. Investigation of Guest-Host Mediated Electrocyclization and Sigma-tropic Rearrangement Reactions

Sigmatropic rearrangements of Trichloroacetimidates and Allylisoureas

A series of cationic [3,3]-sigmatropic rearrangement reactions were attempted in the presence of the assembly (**1**). A special focus was the well-known thermal Overman-rearrangement reaction types. The results of the research performed are summarized in this section.

Short background, Research Ideas and Results.

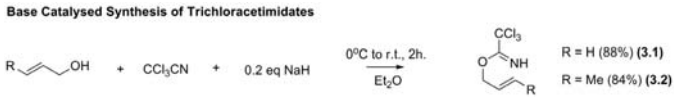
The well known "Overman" sigmatropic rearrangement reactions of trichloroacetimidates and allylisoureas were attempted with the supramolecular assembly (**1**). The cluster-reactions conducted to date turned out to be unsuccessful and are, therefore, only briefly summerized in this sub-chapter.

In the late 1970s, Larry Overman conducted a thorough exploration of the sigmatropic rearrangements of trichloroacetimidates and allylisoureas (Scheme 12)[34-37]. Out of this research a number of versatile and applicable reactions were developed; these sigmatropic rearrangement reactions are still a subject of investigation. For instance, research groups are searching for Lewis acid catalysts that will make these rearrangements run at decreased temperatures[38, 39]. Since these reactions can proceed *via* a cationic transition state we set out to investigate the use of the assembly (**1**) to catalyze/mediate Overman rearrangements, as the idea, depicted in scheme 13.

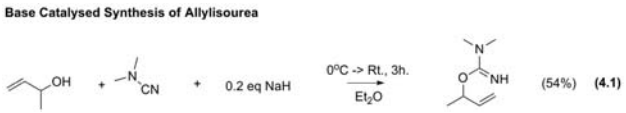
Scheme 12. Thermally sigmatropic rearrangement reaction of Trichloroacetimidates and Allylisoureas.

Scheme 13. The idea of the Overman rearrangement with cluster.

The substrates were readily obtained for these experiments. Both trichloroacetimidates and allylisoureas can be synthesized from the corresponding nitriles and allylalcohols in presence of catalytic base, in this instance sodium hydride. The synthesis of two trichloroacetimidates, (**3.1**) and (**3.2**), are shown in scheme 14, while the synthesis of the allylisourea (**4.1**) is depicted in scheme 15.

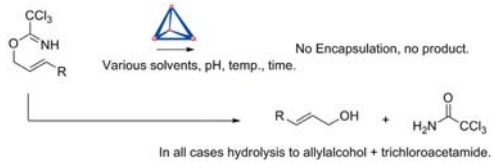


Scheme 14. Synthesis of Trichloroacetimidates (**3.1**) and (**3.2**).



Scheme 15. Synthesis of Allylisourea (**4.1**).

The substrates (**3.1**), (**3.2**) and (**4.1**) were evaluated for enhanced reactivity via binding in (**1**) using similar experiments to those conducted in the previous section and monitored by ^{1}H-NMR. Reactions were performed using both, catalytic and stoichiometric amounts of substrate, and parameters such as pH, temperature, and cosolvents were varied. Unfortunately the trichloroacetamidate substrates are not encapsulated by the assembly and no sigmatropic rearrangement was observed in any of the reactions. Additionally the substrates were unstable in aqueous solutions and hydrolyzed over time in all cases (scheme 16).



Scheme 16. Cluster experiment with trichloroacetimidates (**3.1**) and (**3.2**).

The same experiment with the synthesized allylisourea (**4.1**) showed clear encapsulation in the NMR experiment (one example of a NMR experiment is depicted on figure 6). Consequently the otherwise well defined aromatic cluster peaks become disordered possibly as a result of a break of the assembly symmetry.

Allthought this experiment gave promising encapsulation of substrate – the reference experiment (substrate without cluster) showed no difference – which concludes that the assembly in this case do not catalyze nor mediate the proposed sigmatropic Overman rearrangement.

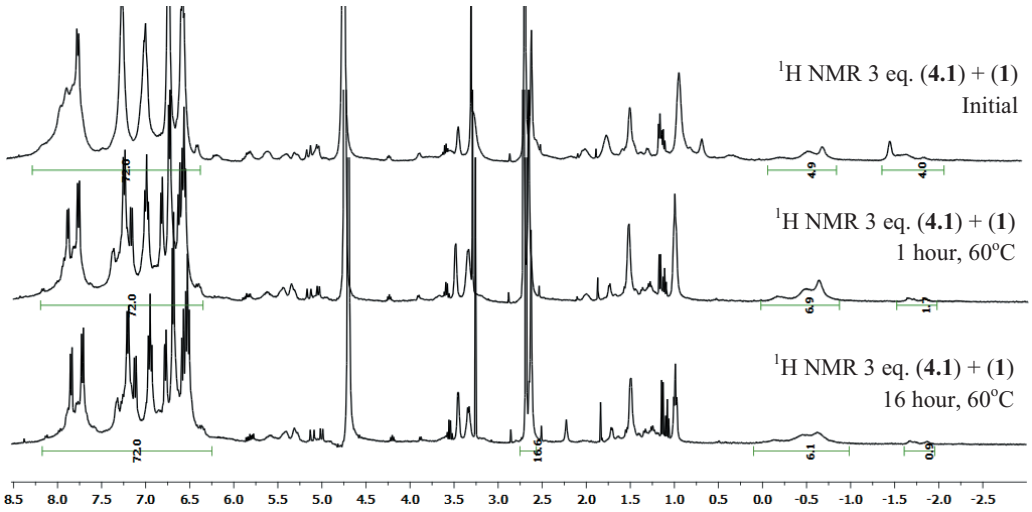
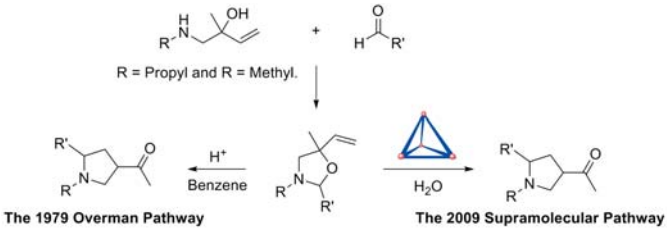


Figure 6. ^{1}H NMR experiment using 3 eq. of the allylisourea (**4.1**) in D_2O at pD = 10, followed over time at 60°C.

Aza-Cope Mannich of Oxazolidines

Short resumé

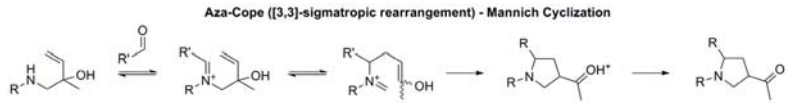
During the exploration of stoichiometric and catalytic reactions that could be mediated by the supramolecular cluster $[Ga_4L_6]^{-12}$ we have found that 5-vinyl-oxazolidines undergo the aza-Cope-Mannich reaction in basic aqueous media to form 3-acetyl-pyrrolidines. This acid-catalyzed reaction was first discovered by Larry Overman in 1979, we hypothesized that this reaction could be performed catalytically in alkaline aqueous solutions (scheme 17). The catalytic reaction is limited to substrates that form products which do not bind more strongly to the cavity of the cluster than the substrates. Our best results were achieved with oxazolidines where R' = H.



Scheme 17. Proposed supramolecular catalysis of oxazolidines to pyrrolidines.

Background

A convenient synthesis of 3-acetyl-pyrrolidines through a sigmatropic rearrangement of homoallylamines containing a allylic hydroxyl group and aldehydes was reported back in 1979 by Overman and Kakimoto (scheme 18).[40] These substrates, upon reacting with an aldehyde to form an iminium ion, undergo a reversible 2-azonia-[3,3]-sigmatropic rearrangement (aza-Cope) followed by a irreversible intramolecular trapping of the iminium with the newly-formed enol (Mannich reaction).



Scheme 18. Tandem aza-Cope Mannich reaction.

An outstanding feature of 2-azonia-sigmatropic rearrangements is that they normally occur under much milder reaction conditions than Cope rearrangement of 1,5-dienes. Due to its usefulness, this reaction has gained attention from synthetic organic chemists and has been reported to be a versatile method for the synthesis of poly-substituted 3-acetyl-pyrrolidines.[41, 42] Furthermore, Overman employed the aza-Cope Mannich approach in his Strychnine total synthesis in 1993[43, 44]. It has been proposed that this reaction is irreversible due to the formation of the carbon-carbon bond in

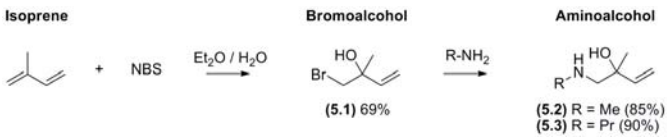
the Mannich cyclization step. Alternative sigmatropic rearrangement reactions typically require an enthalpic driving force, such as formation of aryl-alkene conjugation in *e.g.* benzoheterocyclic compounds[40, 45], or as described earlier in this sigmatropic rearrangement section; rearrangements that form carbonylic compounds from imines (trichloro-acetimidates[34, 36, 37] and allylic pseudoureas[35]).

The aza-Cope Mannich reaction normally occurs at temperatures ranging from room temperature to 80°C. Overman *et al* found the reaction to proceed very well using the ammonium salt of the homoallyl aminoalcohol and an aldehyde in refluxing benzene for 5 hours. Overman *et al.* also found that if an aldehyde was refluxed with the free base of the homoallyl aminoalcohol in benzene the condensed 5-vinyl-oxazolidine could be isolated in very good yields. These isolated oxazolidines also give the desired pyrrolidine product in the presence of an organic acid catalyst.

From earlier reports, it is known that the cluster recognizes and stabilizes iminium ions in water,[46] catalyzes sigmatropic aza-Cope rearrangements from enamines[21, 23] and is able to perform proton-mediated reactions in basic solutions[24-28]. Literature precedent suggests that this aza-Cope Mannich reaction could be performed with the cluster.

Results and discussion

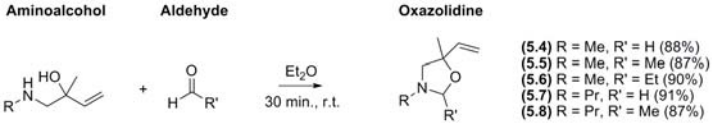
The substrates for the aza-Cope Mannich reaction are readily available and procedures for their synthesis have been published[47]. The synthesis of homoallyl aminoalcohols (**5.2**) and (**5.3**) can be performed in a two step synthesis starting from isoprene. In a mixture e of Et_2O, H_2O, upon mixture of stoichiometric amounts of *N*-bromosuccinamide and isoprene, the bromoalcohol (**5.1**) is formed selectively and can be purified by a fast distillation through a Vigreux column. Two aminoalcohols was synthesized from (**5.1**). Treatment of the latter compound with neat amine (propylamine) yielded the aminoalcohol (**5.3**). Alternatively, reaction with an aqueous methylamine solution yielded (**5.2**). The overall reactions are outlined in scheme 19.



Scheme 19. Synthesis of homoallyl-aminoalcohols.

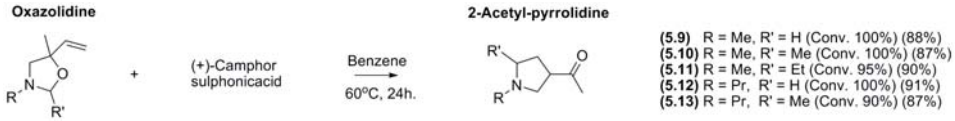
During the course of these studies, it was found that oxazolidines were formed spontaneous upon mixing of the reagents in aqueous solutions. (This was a fact even though the initial idea for the aza-Cope Mannich reaction where recognition of the iminium formed by (**5.3**) and a ketone/aldehyde by the assembly, see scheme 22.)
When a solution of (**5.2**) or (**5.3**) in Et_2O are added to a slight excess of an aldehyde they form the corresponding oxazolidines within minutes at room temperature. After

an aqueous wash of the reaction mixture the oxazolidines were isolated cleanly as clear liquids and could be used directly as substrates without further purification. The overall reaction for the oxazolidine condensation is outlined in scheme 20.



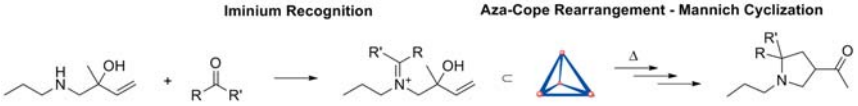
Scheme 20. Condensation of homoallyl-aminoalcohols to oxazolidines.

As a point of comparison, the synthesis of 3-acetyl-pyrrolidines from the oxazolidines (**5.4** – **5.8**) was achieved by treating them with camphor-sulfonic acid (the Overman procedure).[47] The products of these experiments were fully characterized and employed as standards for the assembly-catalyzed reactions. The individual oxazolidines and stoichiometric amounts of acid were heated in benzene for 24 hours at 60°C. The experiments were successful and provided the products (**5.9** – **5.13**) in a good yields (scheme 21). The product (**5.13**) was the only compound that could not be fully characterized by NMR as the conversion observed by GC-MS was only 90%. Products containing R' = alkyl were obtained as a mixture of diastereomers (see experimental section for more detail regarding diastereomeric ratios).



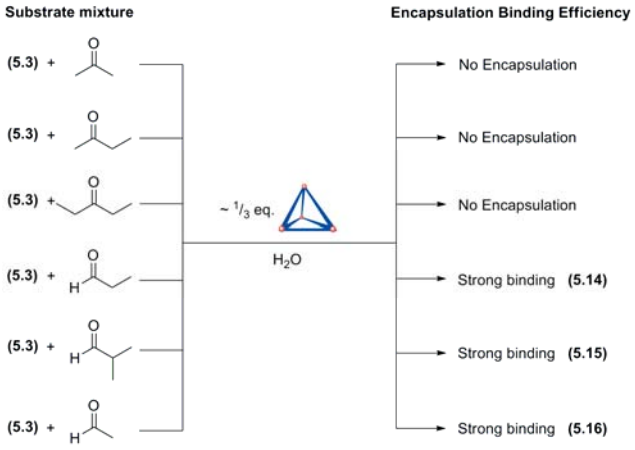
Scheme 21. Acid mediated aza-Cope Mannich of oxazolidine.

Studies were undertaken to assess whether compounds (**5.2** - **5.3**) underwent encapsulation with the assembly (**1**). The hypothesis is represented schematically in scheme 22. From previous studies it is expected that there will be a limitation to the physical size of the substrates that can be encapsulated (approximately 10 – 12 atoms), as a result the first substrate studied was (**5.3**).



Scheme 22. The initial idea – Iminium recognition followed by aza-Cope-Mannich.

The first encapsulation experiments for the aza-Cope Mannich reaction were performed in parallel by mixing the assembly (**1**), and a small range of different carbonyl compounds and the homoallyl-aminoalcohol (**5.3**) (3 uL). The overview of these experiments is listed in scheme 23.



Scheme 23. Parallel encapsulation screening of (**5.3**) + carbonyl compounds.

Experiments where the aminoalcohol (**5.3**) were mixed ketones and the assembly (**1**) showed no encapsulation. These reactions were all followed over time and were also heated to 60°C, but neither reactivity nor encapsulation was observed by ^{1}H NMR spectroscopy. In contrast, when aldehydes were mixed with (**5.3**) an almost instant reaction was physically observed as indicated by the formation of a cloudy solution/suspension. When analyzed by ^{1}H NMR these samples showed a very clear encapsulation with at strong binding efficiency (integration *versus* cluster peaks). The NMR spectra of the encapsulated peaks are shown on figure 7.

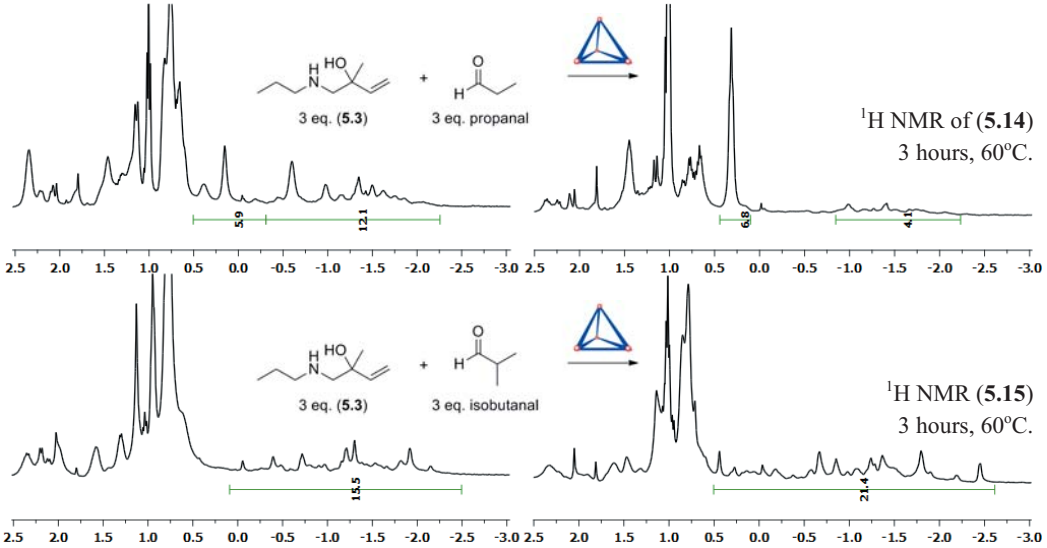


Figure 7. (*top left/right*) ^{1}H NMR in the area 2.5 to -3.0 ppm of reaction (**5.14**) (in H_2O), shows clear encapsulation of compound, integration of ~ 18H, reveals a strong binding of substrate. After heating for 3 hours, the spectrum changes significantly. (*bottom left/right*) ^{1}H NMR of reaction (**5.15**), integration of ~ 16H shows a strong binding of substrate. This again shows significantly changes in the encapsulated material.

Interestingly the two reactions shown in figure 7 both express reactivity of the initial encapsulated compounds, and also that they differ in the binding efficiency of the product formed. By unknown reasons the product(s) formed in (**5.14**) bind less efficient than the product(s) in reaction (**5.15**). Structurally the only difference between the two reactions is the methyl-group in the aldehyde but still the integrals of the encapsulated compound are significantly higher in (**5.15**).

Two following experiments confirmed that the initially encapsulated compounds were in fact oxazolidine. These compounds are encapsulated as neutral guests formed spontaneously from the condensation of the aminoalcohol (**5.3**) and aldehydes.

The ^{1}H NMR spectra (encapsulated area) of the cluster reaction (**5.16**) and oxazolidine substrate (**5.8**) + cluster are depicted in figure 8. The initial ^{1}H NMR was acquired after the standard equilibration time of 15 minutes; subsequently the NMR samples were heated for 60°C in 4 hours. As it appears from the spectra, the encapsulated compounds (starting material and product) are in both reactions identical.

This suggests that the resting state of the encapsulated starting material is the oxazolidine heterocycle and not the iminium ion formed by the aminoalcohol and carbonyl.

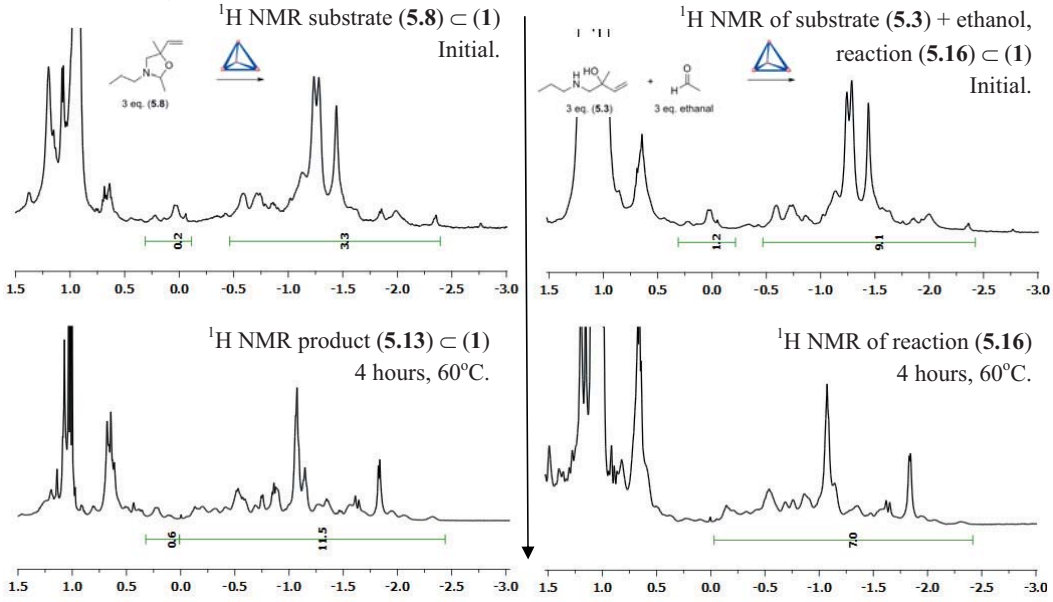


Figure 8. (*left, top and bottom*) The experiment showing ^{1}H NMR spectra of initial encapsulation of 3 eq. oxazolidine (**5.8**) with the assembly (**1**) and the product of reaction (**5.13**) encapsulated after 4 hours at 60°C. (*right, top and bottom*) The experiment showing ^{1}H NMR spectra of encapsulated substrate and product from reaction (**5.16**) with 3 eq. aminoalcohol (**5.3**) and 3 eq. ethanal with the assembly (**1**).

The conclusions derived from these experiments are that;

(i) oxazolidines spontaneous condense with aldehydes and aminoalcohols in water,

(ii) oxazolidines encapsulate as a neutral guest, possible due to an entropically favorable encapsulation into the lipophilic cavity of the cluster (hydrophobic effect),

(iii) upon heating of reaction mixtures new product(s) forms within the cavity of the cluster which were identified to be the corresponding pyrrolidine.

The product formed in the two reactions from figure 8, was identified by GC-MS as 2-acetyl-5-methyl-N-propylpyrrolidine. The product was compared to the synthesized pyrrolidine (**5.13**), both retention time and fraction pattern was identical. Too small amounts of product, impurities from the extraction of product and the complexity in the product NMR made identification of these by NMR impossible at this time. Consequently oxazolidines were, from this point and on, chosen as substrates.

In the first NMR experiments catalytic turnover was not observed or just barely observable. Subsequent studies suggested that this observation was possibly due to a strong binding of the product to the cluster (product inhibition of catalysis).

Substrates synthesized from homoallyl-aminoalcohols (**5.2** and **5.3**) with formaldehyde to give the oxazolidines (**5.4** and **5.7**) did, on the other hand, not suffer from product inhibition.

Those two substrates were surprisingly converted into the corresponding pyrrolidines by a catalytic amount of assembly. The oxazolidines (**5.4** and **5.7**) also showed a decreased initial binding constant compared to substrates derived from other aldehydes. This lower binding efficiency of both substrate and product remains unexplained, but it indicates a fast exchange between the interior and exterior environment of the assembly. A possible explanation could be that these substrates are less lipophilic.

In figure 9 (on the next page), one example of a catalytic reaction with the substrate (**5.7**) is illustrated showing the substrate decrease and product increase.

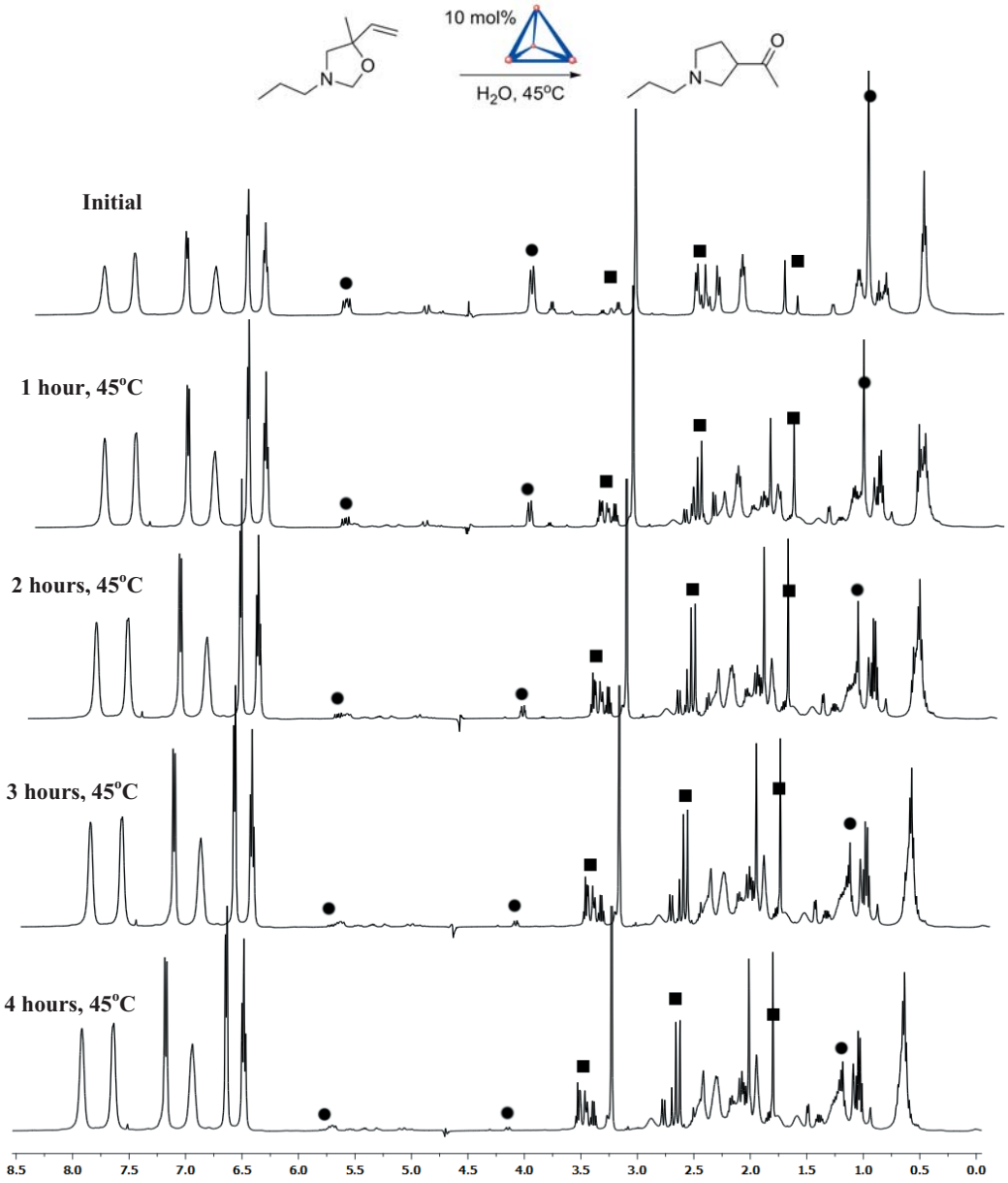


Figure 9. ^{1}H NMR of catalytic reaction of oxazolidine (**5.7**) with 10% of assembly (**1**). Reaction were followed in unbuffered H_2O every hour by NMR with use of the Watergate solvent suppression pulse program.

● Signs mark the disappearence of substrate (the two signs on the left side is disappearance of vinyl peaks and on the right side the methyl).

■ Marks the increase of product (right the methyl α to carbonyl and left the CH α to carbonyl.).

Part III. Investigation of Guest-Host Mediated Electrocyclization and Sigma-tropic Rearrangement Reactions

As it appears from the catalytic experiment(s) the reaction is more than 95% complete after 4 hours with the use of 10% catalyst loading in unbuffered H_2O. This reaction was however left for 24 hours at 45°C, for total completion, extraction with $CDCl_3$ and identified as the same pyrolidine as formed in the earlier mentioned original "Overman" acid mediated aza-Cope Mannich product. The ^{13}C NMR of the products are shown on figure 10.

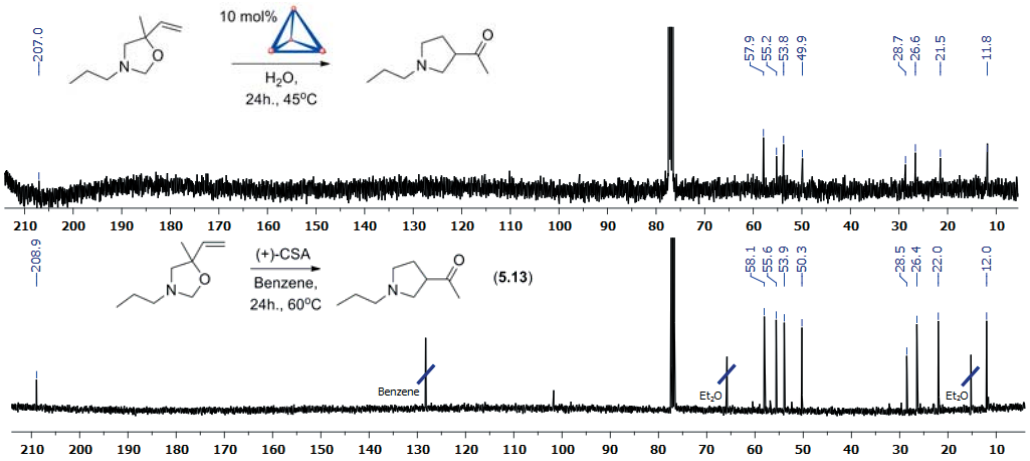


Figure 10. (*top*) ^{13}C NMR spectrum from $CDCl_3$ extraction of the reaction between (**5.7**) and 10 mol% (**1**), reaction was extracted after 24 hours at 45°C. The first 4 hourly ^{1}H NMR spectra are shown on figure 9. (*bottom*) ^{13}C NMR spectrum of 3-acetyl-1-propyl-pyrrolidine (**5.12**) synthesized with acid ((+)-camphor sulphonic acid) from corresponding oxazolidine.

For assurance that the aza-Cope Mannich is performed by the cluster and not by other factors, *e.g.* ion strength or binding to the exterior of the assembly, a competitive inhibition experiment was conducted. In these experiments you use a known good guest to block the cavity, therefore we used 2 eq. tetraethylammonium (Et_4N^+) as the inhibitor and performed the same reaction and followed its course by ^{1}H NMR spectroscopy. A point kinetic log-plot of the integration of substrate is shown on figure 11.

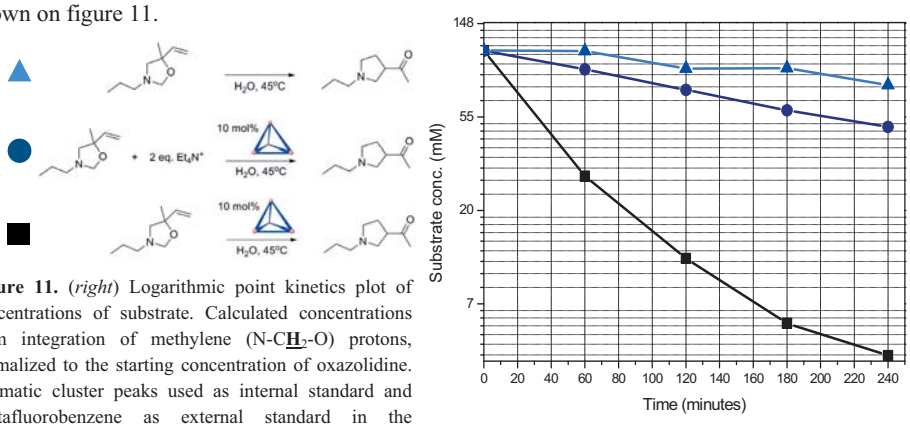


Figure 11. (*right*) Logarithmic point kinetics plot of concentrations of substrate. Calculated concentrations from integration of methylene (N-C**H**$_2$-O) protons, normalized to the starting concentration of oxazolidine. Aromatic cluster peaks used as internal standard and pentafluorobenzene as external standard in the background reaction.

The plot of substrate concentration *versus* time clearly shows the assembly catalyzes the aza-Cope Mannich reaction. The initial rate, of the cluster catalyzed reaction, is fast and after 1 hour at 45°C the conversion of the substrate to product is > 70%. The inhibition of the cluster with 2 eq. of Et_4N^+ also slows down the reaction as we hoped, but as the plot also reveals, the conversion-rate is in this experiment is faster than the experiment in H_2O alone. This suggest either competitive inhibition or that the ion strength / exterior of the assembly have some effect on the reactionrate. The background reaction was placed at 45°C for a total of 18 hours and submitted to GC-MS for analysis of the compounds. This experiment showed two reaction pathways for the substrate in unbuffered solution; it either forms the pyrrolidine or hydrolyzes back to the aminoalcohol (**5.3**). The partition between the compounds in the sample was at 18 hours: Oxazolidine (30%), Pyrrolidine (39%) and Aminoalcohol (30%), which must be considered a significantly amount of hydrolyzed and also formed product, figure 12.

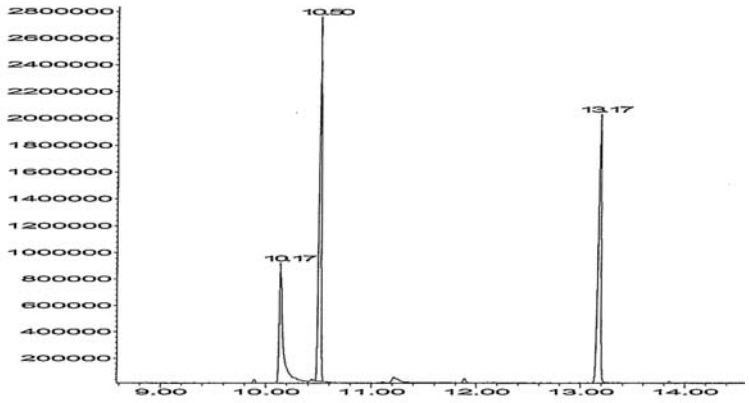
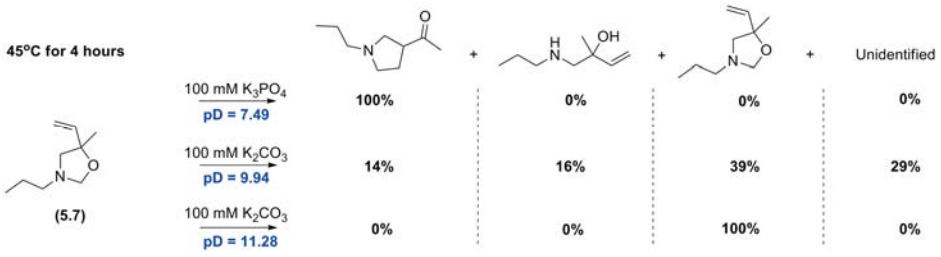


Figure 12. CG-MS trace from the background reaction in H_2O (18 h., 45°C). First peak at rt = 10.17 min. corresponds the aminoalcohol, second peak at rt = 10.50 min. is the oxazolidine and last peak at 13.17 corresponds to the pyrrolidine product.

To overcome the background hydrolysis and formation of product, the oxazolidine (**5.7**) was subjected to 3 different buffered D_2O solutions. On scheme 24 the results for the pD experiments are listed.



Scheme 24. Conversion of (**5.7**) in buffered solutions at different pD values measured by GC-MS.

With regards to hydrolysis, this oxazolidine seams to be more stable at higher pD/pH values and therefore no ring-opening of oxazolidine, hydrolysis or sigmatropic rearrangements were observed under these conditions. At 4 hours at 45°C, no degradation of the starting material was observed at the high pD of 11.28. We set out to investigate the cluster experiments at this high pH.

Four experiments were performed to investigate the pH effect on the catalytic and background hydrolysis reactions. These reactions were setup same way as the previous point kinetic reactions and the results are plotted on figure 12.

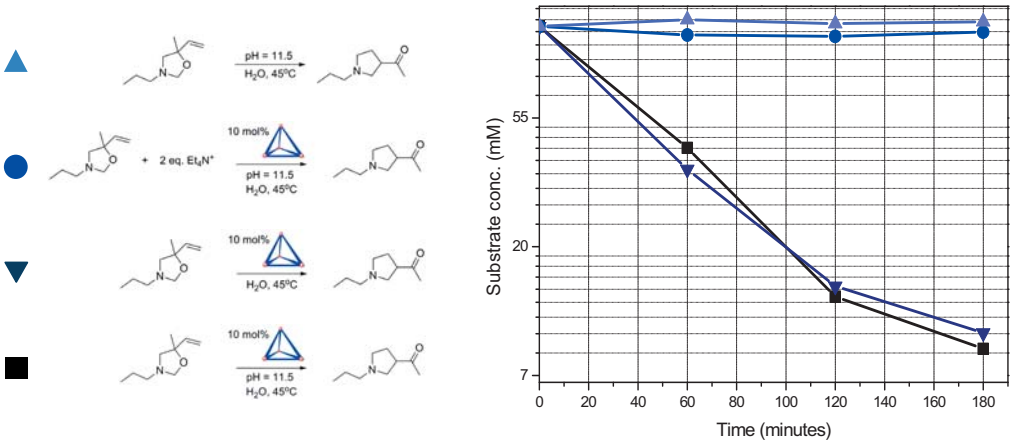


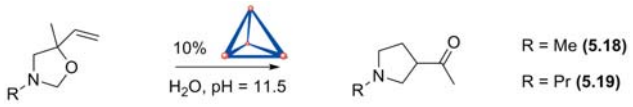
Figure 13. Logarithmic point kinetics plot of concentrations of substrate versus time.

The effect of pH is compared in buffered (100 mM K_2CO_3 at pH=11.5) and unbuffered (H_2O) solvent in the catalytic reaction of compound (**5.7**).

Calculated concentrations from integration of methylene (N-C**H**$_2$-O) protons, normalized to the starting concentration of oxazolidine. Aromatic cluster peaks used as internal standard (integrated to 72H) in the cluster experiments and pentafluorobenzene as external standard (sealed tube) in the background reaction.

The reaction was followed over 3 hours at 45°C and the plot of the integration clearly shows the effect of reaction at this buffered high pH solvent. The background reaction and the reaction with the use of 2 eq. of inhibitor show nearly no decrease in substrate in this time-frame which means a non-significant hydrolysis takes place. In the catalytic reactions, the comparison of the buffered and un-buffered looks almost identical. This suggests that the rate of this catalytic aza-Cope Mannich reaction is independent of the proton concentration (although we know it is, of course). These experiments are, however, only preliminary point kinetic studies and a more thorough study of the pH influence on the reaction rate must be completed before any conclusive statements can be made. The reaction is proposed to go through an initial protonation (cationic recognition of the substrate) followed by ring opening of the oxazolidine. One would therefore imagine that proton concentration may play a significant role in the reaction rate.

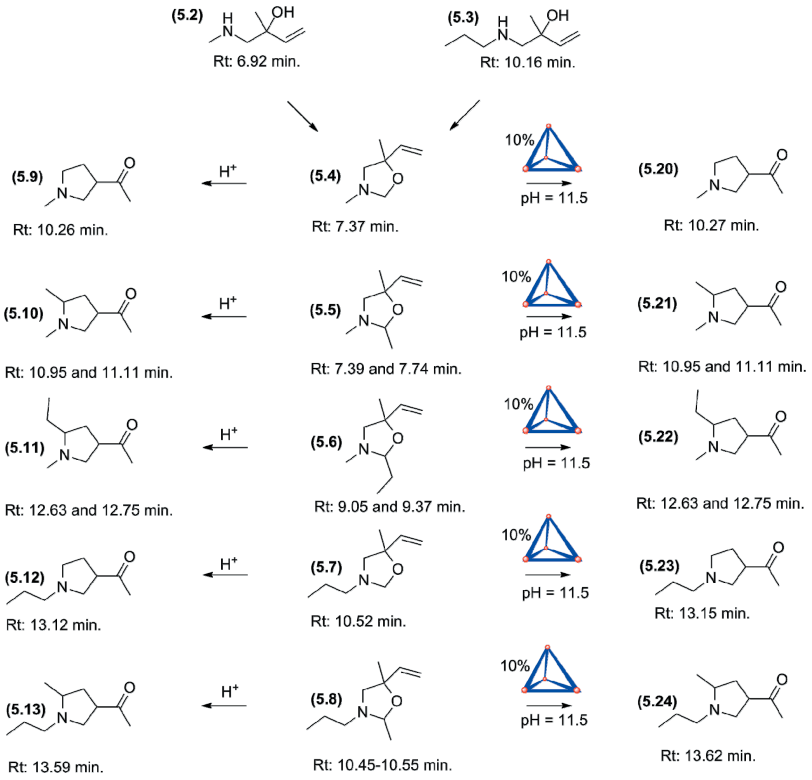
For the two substrates that showed fast and clear catalytic reactivity with the cluster, two larger scale reactions were conducted in order to fully characterize the reaction product. Unfortunately the reaction was not all complete after 4 hours at 45°C when the reactions were extracted; remaining peak resonances from the starting material were present in the NMR spectrum. Data were compared with literature values, the pyrrolidines synthesized earlier and was this way identified. On scheme 25, the two reactions are depicted.



Scheme 25. The two upscale reactions performed on the substrates with fastest and efficient turnover.

All the synthesized oxazolidine substrates were tested for catalytic activity with the cluster. From these experiments it was found that alkyl substitution on the 2-position had a drastic impact on the reaction rate.

In scheme 26, the reactions are tabulated and the retention time of the products listed. In the experimental section details of the reaction condition and conversion/turnover of the substrate listed.



Scheme 26. Overview of the compounds, catalytic cluster experiments and proton mediated together with the retention time from the CG-MS track for identification and conversion calculation.

Conclusions to the aza-Cope Mannich reaction

The results from aza-Cope Mannich reactions performed with the $[Ga_4L_6]^{-12}$ supramolecular cluster can be summarized with the following points.

Allylaminoalcohol (**5.3**) spontaneously forms oxazolidines when mixed with aldehydes (**5.14 - 5.16**) in aqueous solutions, these neutral heterocycles encapsulate in the cluster. Upon heating a transformation of the substrate encapsulated within the cluster cavity occur. The same substrate (**5.3**) does not react with ketones.

Oxazolidines can be easily synthesized (**5.4 - 5.8**) and be used as substrates in cluster experiments to form pyrrolidines (**5.20 - 5.24**).

Oxazolidines are encapsulated in the assembly, where they protonate, ring-open and undergo the aza-Cope Mannich reaction; this reactivity can be turned off by the addition of inhibitor (Et_4N^+) to the reaction for prevention of substrate encapsulation.

When the oxazolidines ((**5.4**) and (**5.7**)) are formed with formaldehyde, the transformation from oxazolidine to pyrrolidine occurs much faster and catalytically. In contrast to the oxazolidines substituted on the 2 position, where slow turnover was observed.

At higher pH the background hydrolysis reaction decrease and the cluster experiments seems unaffected at this pH.

Experimental Section

General

Unless otherwise indicated, all operations were performed under anhydrous conditions and inert atmosphere employing standard Schlenk techniques under nitrogen. Glassware was dried in an oven at 160^{o}C overnight or flame-dried prior to use.

GC-MS analysis was performed on a Agilent Technologies 6890N gas chromatograph with a Agilent Technologies 5973 electron-impact mass-spectrometry detector equipped with a Agilent DB-5ms, 0.25mm * 30 m * 0.5 um (catno. 122-5536) column. A standard method were in general used with a temperature gradient 50^{o}C (3 min, 0^{o}C/min), 200 ^{o}C (10 ^{o}C/min), 300 ^{o}C (15 ^{o}C/min), 300 ^{o}C (0^{o}C/min, 5 min) with a gas flow of 1.2 ml/min.

NMR spectra were acquired using Bruker AV-300, AVQ-400, AVB-400 and AV-500 spectrometers. Chemical shifts are reported as part per million (ppm, δ) and ^{1}H and ^{13}C chemical shifts are referenced to the corresponding residual protic solvent resonance. Signal multiplicity and shape are reported using the following abbreviations: s, singlet; bs, broad singlet; d, doublet; t, triplet; q, quartet; m, complex multiplet. Measurements of ^{1}H NMR shifts performed in H_2O as solvents were acquired using a Watergate solvent suppression pulse-program. Deuterated solvents were purchased from Cambridge Isotope Laboratories.

All high-resolution mass spectra (HRMS) were recorded at the University of California, Berkeley Microanalytical Facility with electrospray ionization detection in positive mode.

Solvents for cluster experiments were degassed prior to use by bubbling N_2 gas through the solvent. Aprotic solvents used for synthesis of substrates were anhydrous and purified by passing through a column of activated alumina under nitrogen pressure.

All reagents were obtained from commercial suppliers and used without further purifications unless stated otherwise.

Synthesis of The Assembly

(0.1) 2,3-dimethoxybenzoyl chloride: (NB: This is a reproduced procedure)[48]. In a flame dried Schleck-flask 2,3-dimethoxybenzoic acid (4.435 g, 24.43 mmol) was slowly added $SOCl_2$ (10 mL, 16.3 gr., 137 mmol) at 0°C and under N_2. The solution was allowed to warm to room temperature and the resulting red-orange homogenous solution was stirred night-over (16 h.). The volatiles were then removed under reduced pressure and the resulting crystalline off-white powder was to added DCM (20 mL) stirred for 20 minutes and solvent were stripped off. This was performed twice before the compound was dried under reduced pressure to yield approximately 4.9 grams of white crystalline product. The compound was used as is in following reaction, assuming quantitative yield.

(0.2) N,N'-Bis(2,3-dimethoxybenzoyl)-1,5-naphtylenediamine: (NB: This is a reproduced procedure[10].) In the same dry Schleck-flask cooled to 0°C, the freshly prepared 2,3-dimethoxybenzoyl chloride (**0.1**) (4.9 gr., 24.4 mmol) and 1,5-diamino-naphtalene (1.60 g, 10.1 mmol) was dissolved in DCM (40 mL). An excess of Et_3N (5 mL) was slowly added, immediately forming white precipitate (Et_3NHCl). The suspension was then stirred at 0°C for three hours. Additional DCM (40 mL) was added to the mixture, which was washed with water (100 mL), then dilute HCl (100 mL, 1 M) followed by a dilute NaOH solution (100 mL, 1M). The organic layer was dried over $MgSO_4$, filtered and concentrated on a rotary evaporator. Diethyl ether (20 mL) was added and the precipitate was collected by filtration and the product was air dried. This yielded tan powder (3.84 gr., 7.9 mmol, 78 %). The ^{1}H-NMR was compared to the described in literature.

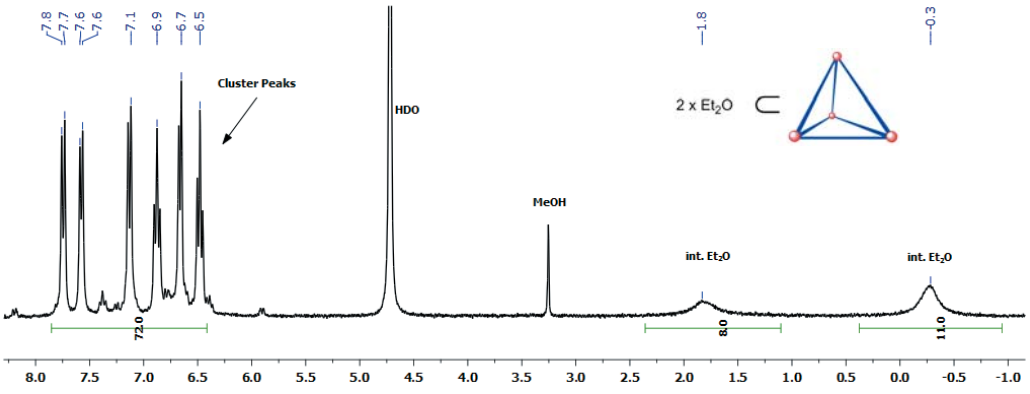
^{1}H NMR (300 MHz, *$CDCl_3$*) δ ppm 10.75 (s, 2H, NH), 8.56 (d, *J* = 7.6 Hz, 2H), 7.89 (dd, *J* = 8.1, 1.5 Hz, 4H), 7.60 (t, *J* = 8.1 Hz, 2H), 7.27 (t, *J* = 8.1 Hz, 4H), 7.16 (dd, *J* = 8.1, 1.5 Hz, 2H), 4.09 (s, 6H, OMe), 3.99 (s, 6H, OMe).

(0.3) N,N'-Bis(2,3-dihydroxybenzoyl)-1,5-naphtylenediamine = L: (NB: This is a modified procedure described[10].) In a flame dried Schlenk-flask (**0.2**) (2.108 gr., 4.33 mmol) was dissoluted in DCM (30 mL) and cooled to -78 °C. This mixture was then slowly charged with BBr_3 (8.5 mL, 90 mmol) *via* a syringe. The reaction mixture was allowed to warm to room temperature and stirred for 5 days for to achieve full demethyllation[49]. The reaction mixture was then quenched with ice (250 gr.) in a round bottomed flask cooled in an ice bath. A notably intense reaction was observed while quenching. The remaining suspension was placed on a rotorary evaporator and the volatiles (DCM) were removed gently under reduced pressure. It was then heated up to 90°C at atmospheric pressure and stirred heavily for 3 hours. The solids were collected by filtration, followed by washing with H_2O (50 mL x5), MeOH (50 mL x 5) and last Et_2O (50 mL x 2). The product was dried under vacuum night over to give a pale-tan off-white powder (1.303 gr., 3.03 mmol, 70%). ^{1}H NMR of this was compared to the described literature.

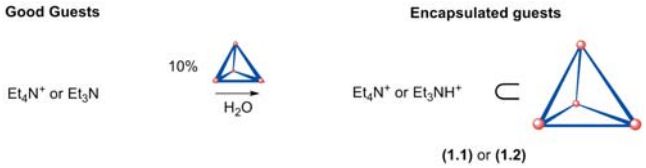
^{1}H NMR (300 MHz, *d6-DMSO*) δ ppm 11.89 (bs, 2H), 10.92 (s, 2H), 9.62 (bs, 2H), 7.92 (d, *J* = 8.4 Hz, 2H), 7.86 (d, *J* = 7.5 Hz, 2H), 7.65-7.59 (m, 4H), 7.03 (d, *J* = 7.5 Hz, 2H), 6.84 (t, *J* = 7.8 Hz, 2H).

(1) $K_{12}[(Et_2O)_2 \subset Ga_4L_6]$: (NB: This is a procedure previously described[12]): In a flame dried shleck-flask the ligand (**0.3**) (1.3 gr., 3.02 mmol) was suspended in MeOH (150 mL) and degassed by 3 vacuum / N_2 cycles. This was added KOH (0.504 M in MeOH, 17 mL, 8.57 mmol) and degassed again by 3 vacuum / N_2 cycles. To the resulting homogenous solution was added [$Ga(acac)_3$] (739 mg, 2.01 mmol) and KCl (61 mg, 0.82 mmol) and the resultant mixture was subsequently degassed 3 more times by vaccum/N_2 cycles. The reaction was stirred for 4 hours before it was reduced to a third of the volume under vacuum. The assembly (**1**) was precipitated out by the dropwise addition of Et_2O (approx. 30 mL) and isolated by filtration on a medium frit under a flow of nitrogen. The product was washed with Et_2O (20 mL x 5), pentane (20 mL x 4) and again with Et_2O (20 mL x 3) followed by drying overnight on the vacuum-line to yield a yellow powder (1.497 gr., 83%). The product was stored under nitrogen in a glovebox until used. A peak of methanol was observed, this could not be removed by vacuum over time and this is associated with the highly polarized ionic cluster. The molecular weight of the cluster is in further experiments standardized to 3600 DA, therefore giving a yield of 83%.

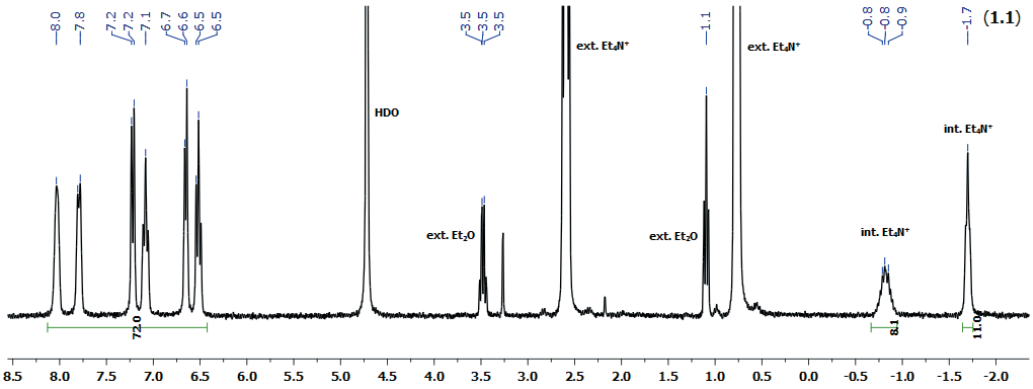
^{1}H NMR (300 MHz, *D_2O*) δ ppm 7.73 (d, J = 8.5 Hz, 12H, *aryl*), 7.56 (d, J = 7.4 Hz, 12H, *aryl*), 7.11 (d, J = 8.1 Hz, 12H, *aryl*), 6.86 (d, *J* = 8.1 Hz, 12H, *aryl*), 6.64 (d, *J* = 7.1 Hz, 12H, *aryl*), 6.46 (t, J = 7.6 Hz, 12H, *aryl*), 3.24 (s, 2H, MeOH, *exterior*). Guest: 1.80 (bs, 8H), -0.30 (bs, 12H).



(1.1) $[Et_4N^+ \subset Ga_4L_6]^{-11}$: In a small vial (**1**) (20 mg) was mixed in a standard degassed solution of Et_4NBr (approx. 10 eq.) in D_2O (500 uL). The sample was shaken and NMR acquired after 30 min.

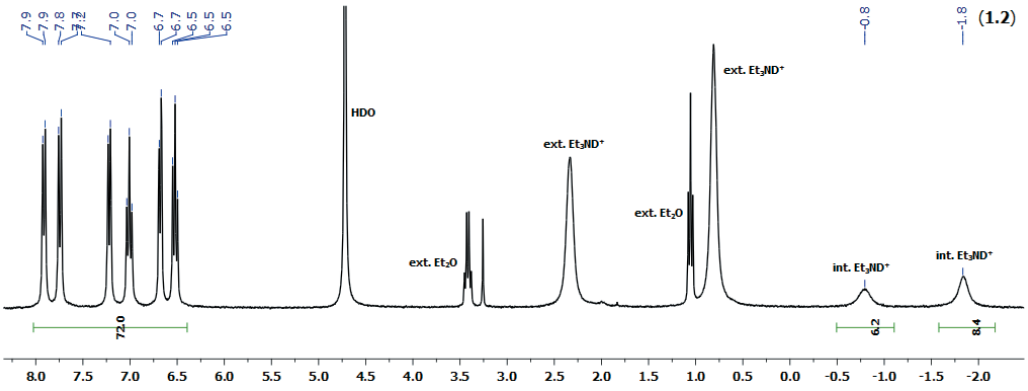


^{1}H NMR (300 MHz, *D_2O*) δ ppm 8.09-7.96 (m, 12H, *aryl*), 7.85-7.74 (m, 12H, *aryl*), 7.20 (d, J = 8.1 *Hz*, 12H, *aryl*), 7.06 (t, *J* = 8.1 *Hz*, 12H, *aryl*), 6.63 (d, *J* = 7.2 *Hz*, 12H, *aryl*), 6.49 (t, J = 7.8 *Hz*, 12H, *aryl*), 3.46 (q, J = 6.9 *Hz*, 6H, Et_2O *exterior*), 3.24 (s, 2H, MeOH *exterior*), 2.57 (q, J = 7.2 *Hz*, 76H, NEt_4^+ *exterior*), 1.07 (t, J = 6.9 *Hz*, 9H, Et_2O *exterior*), 0.75 (t, J = 7.2 *Hz*, 114H, NEt_4^+ *exterior*). Guest: -0.82 (m, 8H, NEt_4^+ *interior*), -1.70 (m, 12H, NEt_4^+ *interior*).



(**1.2**) **$[Et_3NH^+ \subset Ga_4L_6]^{-11}$:** In a small vial (**1**) (20 mg) was mixed in a standard degassed solution of Et_3N in D_2O. The sample was shaken and NMR acquired after 30 min.

1H NMR (300 MHz, *D_2O*) δ ppm 7.89 (d, J = 7.7 Hz, 12H, *aryl*), 7.72 (d, J = 8.5 Hz, 12H, *aryl*), 7.20 (d, J = 8.1 *Hz*, 12H, *aryl*), 6.99 (t, *J* = 8.1 *Hz*, 12H, *aryl*), 6.66 (d, *J* = 7.2 *Hz*, 12H, *aryl*), 6.50 (t, J = 7.8 *Hz*, 12H, *aryl*), 3.40 (q, J = 7.2 *Hz*, 6H, Et_2O *exterior*), 3.24 (s, 2H, MeOH *exterior*), 2.31 (bs, 28H, NEt_3 *exterior*), 1.04 (t, J = 7.2 *Hz*, 9H, Et_2O *exterior*), 0.79 (bs, 42H, NEt_3 *exterior*). Guest: -0.80 (bs, 6H, NEt_3H^+ *interior*), -1.85 (bs, 9H, NEt_3H^+ *interior*).

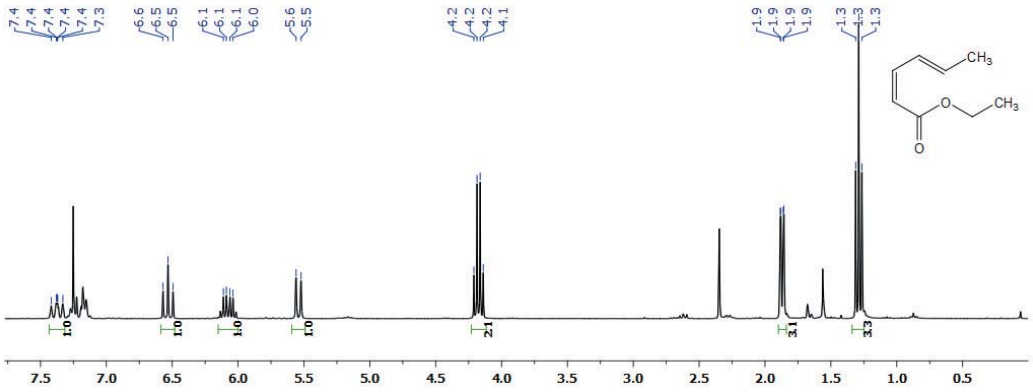


Synthesis of Substrates for Electrocyclization and Sigmatropic Rearrangement

(**2.1**) **(2*Z*,4*E*)-methyl hexa-2,4-dieneoate.** Procedure as described[32]. A solution of Phosphonoester (473 uL, 2 mmol), 18-crown-6 (529 mg, 2 mmol) in 20 ml anhydrous THF was cooled to -78°C under nitrogen and treated with $KN(TMS)_2$ (2 mmol, 0.3M in toluene, tritrated prior to use with fluorene, 2,6-ditertbutyl-4methyl-phenol). Crotonaldehyde (145 uL, 2 mmol) was then added and the resulting mixture was stirred for 45 min at -78°C. A saturated NH_4Cl sol. (30 mL) was added, and the reaction allowed to warm to r.t., and the product was extracted into diethylether (3 x 40 mL). The ether extracts were washed with brine (50 ml), dried ($MgSO_4$), filtered and solvents removed on a rotary evaporator. The product was isolated by flash chromatography. To yield a clear oil (**2.1**) 242 mg (86%).

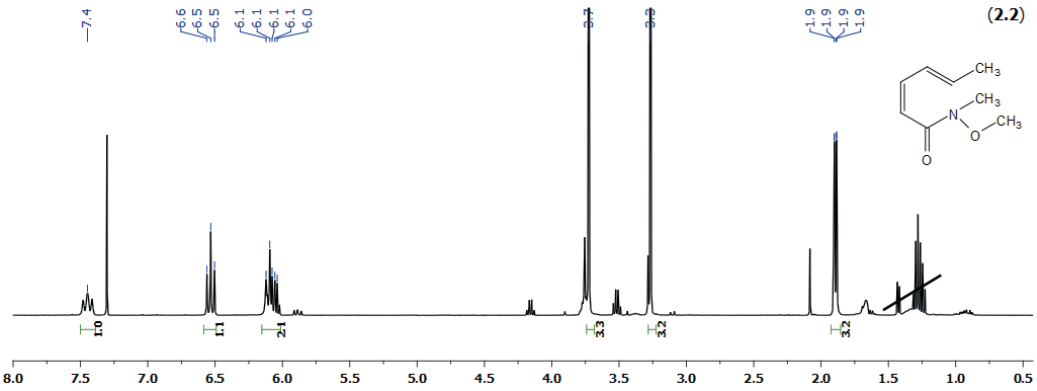
1H NMR (300 MHz, *$CDCl_3$*) δ ppm 7.37 (m, 1H), 6.53 (t, *J* = 11.33 Hz, 1H), 6.08 (qd, *J* = 13.74, 6.85 Hz, 1H), 5.62-5.47 (d, 1H), 4.17 (q, *J* = 5.39 Hz, 1H), 1.86 (d, *J* = 7.97, Hz, 1H), 1.29 (t, 1H). GCMS showed the product: RT = 10.44 min (mz = 140, M^+).

Z:E ratio = 24 based on the NMR dublet at ~ 5.5 (Z - low *J*) and ~ 5.75 (E - High *J*).



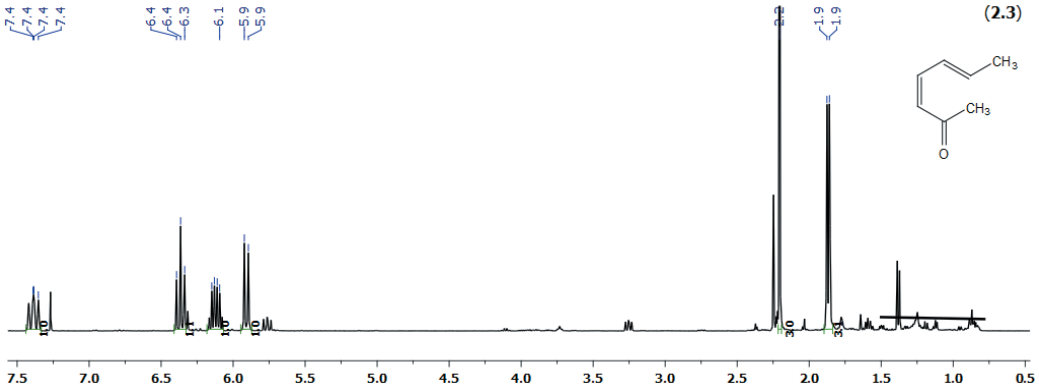
(**2.2**) **(2Z,4E)-N-methoxy-N-methylhexa-2,4-dienamide.** Procedure as described[33]. To a slurry of Me(MeO)NH.HCl (112 mg, 1.25 mmol) and (**2.1**) (112 mg, 1 mmol) in 15 mL of THF at -5°C under nitrogen was added MeMgCl in THF (1.33 mL, 3.0 M, 4 mmol) over 15 min. maintaining the temperature between -2 and -5 °C. After 1h at -5°C the reaction mixture was warmed to 25°C over 1 h, aged overnight (12 hours). The reaction mixture was then quenched into 1 M NH_4Cl (15 mL) then heated to 30-35 °C for 15 min. The resulting mixture was extracted with Et_2O (50 mL x 2) and the combined organic phases dried over $MgSO_4$ and filtered. After removal of solvents on a rotary evaporator the crude product was purified on a silica column to yield the weinreb amide as a pale yellow clear liquid: 101 mg (65%).

1H NMR (400 MHz, $CDCl_3$) δ ppm 7.43-7.36 (m, 1H), 6.48 (t, J = 11.35 Hz, 1H), 6.08-5.98 (m, 2H), 3.66 (s, 3H), 3.22 (s, 3H), 1.85 (d, J = 6.81, 3H). CGMS showed on peak at 13.41 min, m/z = 155 (M^+).



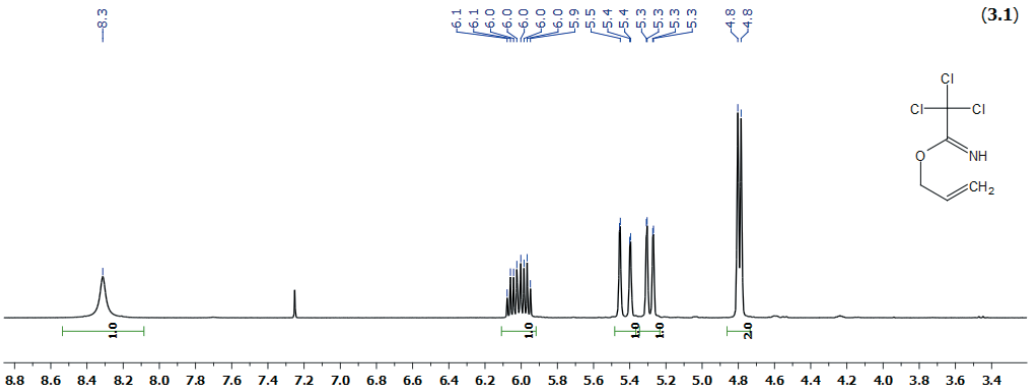
(**2.3**) **3,5-diene-2-heptenone.** A flamedried Slenck flask was charged with THF (20 mL) and (**2.2**) (204 mg, 1.31 mmol). The flask was cooled to -78°C and MeLi was added (1.6 mL, 2.6 mmol, 1.6M). Reaction turned from clear, to deep red / brownish indicating a immediate reaction. The reaction mixture was stirred at this temperature and followed by TLC for the fully consumption of weinreb amide, fully conversion was observed after 15 minutes. The reaction was quenched with a solution of NH_4Cl (20 mL, 1M) and added Brine (10 mL). It was exctracted with Et_2O (2 x 40 mL) and the combined organic layers where dried with MgSO4, filtered and evaporated. The product was chromatographed on a Biotage (column 12-M), and the fractions containing methyl-ketone was evaporated to yield (**2.3**) as yellow oil, 82 mg (56%).

^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.48-7.31 (m, 1H), 6.37 (t, J = 11.32 Hz, 1H), 6.13 (qd, J = 13.99, 6.91 Hz, 1H), 5.92 (d, J = 11.26 Hz, 1H), 2.22 (s, 3H), 1.88 (d, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm 198.9, 143.1, 141.9, 128.9, 122.6, 31.6, 18.7.

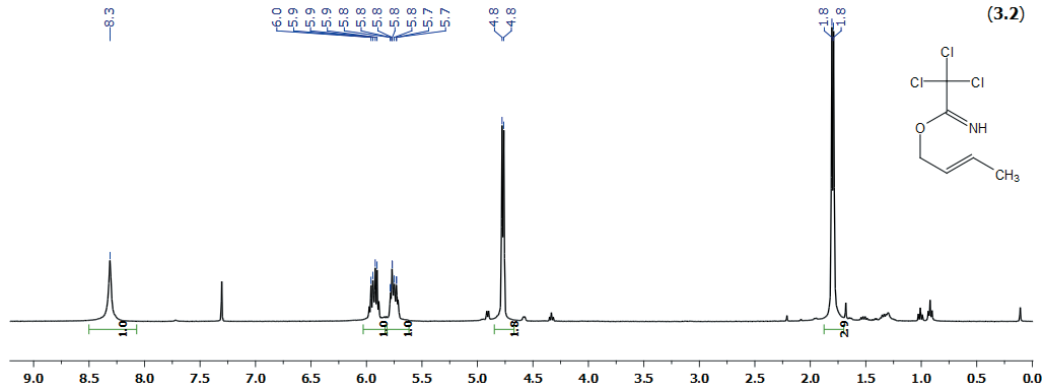


(**3.1**) **Allyltrichloroacetimidate** and (**3.2**) ***trans*-crotyltrichloroacetimidate**. Prepared by described procedure[50]. A flame dried Schlenk flask was charged with anhydrous diethyl ether (10 mL) followed by sodium hydride (24 mg, 1 mmol). To the resulting suspension allyl alcohol ((**3.1**), 680 uL, 10 mmol); (**3.2**), 855 uL, 10 mmol) was added dropwise and after 5 minutes the solids had dissolved (H_2 evaporation). The reaction mixture was then cooled to 0°C with an ice-bath and trichloroacetonitrile (1 mL, 10 mmol) was added dropwise over a period of 5 min. The mixture was allowed to warm to room temperature over an period of 60 minutes and stirred for additional 60 minutes. The reaction was then neutralized with saturated $NaHCO_3$ (10 ml). It was extracted with Et_2O (3 x 10 mL) and the combined organic layers was dried over $MgSO_4$ and concentrated in vacuum on a rotary evaporator.

Yielding (**3.1**) as a clear orange oil: 1.78 gram (88%). ^{1}H NMR (300 MHz, *CDCl$_3$*) δ ppm 8.32 (s, 1H), 6.02 (ddd, *J* = 22.60, 10.68, 5.43 Hz, 1H), 5.43 (dd, *J* = 17.23, 1.47 Hz, 1H), 5.30 (dd, *J* = 10.51, 1.20 Hz, 1H), 4.80 (d, *J* = 5.43 Hz, 2H). ^{13}C NMR (75 MHz, *CDCl$_3$*) δ ppm 162.44 (1C), 131.34 (1C), 118.46 (1C), 91.32 (1C), 77.42 (2C).



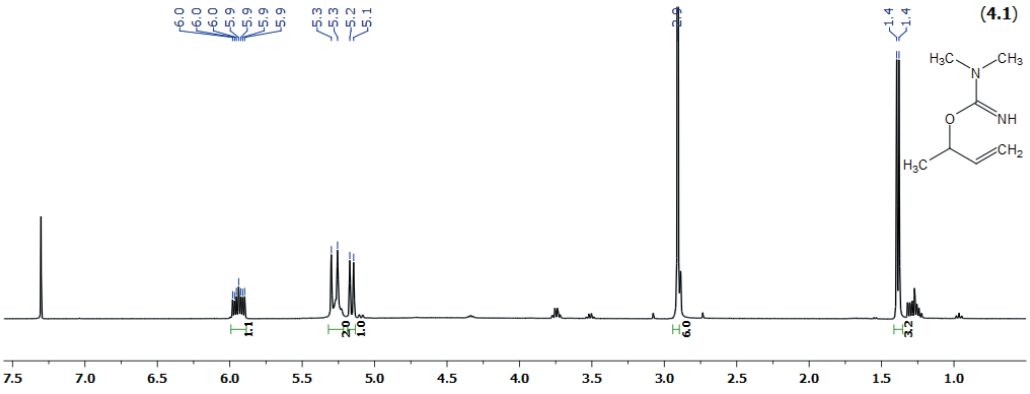
Yielding (**3.2**) as a slightly yellow oil: 1.76 gram (81%). 1H NMR (400 MHz, $CDCl_3$) δ ppm 8.25 (s, 1H), 5.87 (dq, J = 13.1, 6.5, 1H), 5.69 (dtd, J = 7.7, 6.4, 1.3, 1H), 4.71 (d, J = 6.3, 2H), 1.73 (d, J = 6.4, 3H). 13C NMR (100 MHz, $CDCl_3$) δ ppm 162.61 (C), 131.82 (CH), 124.30 (CH), 70.17 (CH2), 18.09 (CH3).



(**4.1**) **2-(1-methyl)-allyl-1,1-dimethylisourea**. This is a modified proceeding described[51]. A solution of Dimethylcyanamide (0.5 mL, 6.13 mmol) and 2-buten-2-ol (0.53 mL, 6.13 mmol) in dry diethylether (10 mL) was added 0.2 eq. sodiumhydride (15 mg, 0.61 mmol). The resulting mixture was stirred at r.t. for 3 h. after which additional DCM was added (20 mL) and the resulting solution was washed with water (3 x 20 mL). The organic phase was then washed with brine (30 mL) and dried over $MgSO_4$, then evaporated to give the product as a clear oil yield: 471 mg (54%).

^{1}H NMR (300 MHz, *$CDCl_3$*) δ ppm 5.89 (m, 1H), 5.24 (d, 1H), 5.22 (bs, 1H), 5.12 (d, 1H), 2.87 (s, 6H), 1.35 (d, 3H). 1H NMR (400 MHz, *8d-toluene*) δ ppm 5.82 (m, 1H), 5.45 (bs, 1H), 5.15 (d, 1H), 4.95 (d, 1H), 2.44 (s, 6H), 1.26 (d, 3H).

^{13}C NMR (100 MHz, *CDCl3*) δ ppm 160.6, 139.0, 114.6, 72.48, 37.2, 20.6. ^{13}C NMR (APT (CH/CH3 +) (C/CH2 -), 100 MHz, *8d-Toluene*) δ ppm 159.3 (-), 139.4 (+), 113.7 (-), 71.7 (+), 36.3 (+), 19.8 (+).

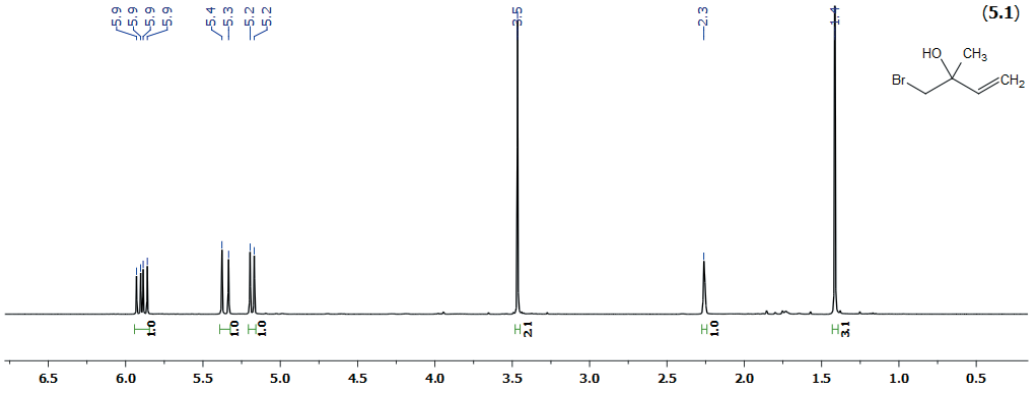


Synthesis of Substrates for aza-Cope Mannich

(**5.1**) **1-bromo-2-methyl-3-butene-2-ol:** (NB: This procedure has previously been described[47]). A mixture of diethyl ether (35 mL), water (25 mL) and isoprene (15 mL, 150 mmol) was rapidly stirred and cooled to 0°C in a flask fitted with at dry-ice condenser. N-Bromosuccimide (26.7 g., 150 mmol) was added in one portion, the cooling bath removed, and reaction was stirred at room temperature for 16 hours. Additional water (20 mL) and diethyl ether (20 mL) was added, the ether phase separated, and the aqueous layer extracted with diethyl ether (3 x 25 mL). The combined organic phases were dried with $MgSO_4$, filtered and the ether removed on a rotary evaporator. The crude product was purified with a quick vacuum distillation through a short column, yielding a very pale-green clear liquid: 14.0 gram (57%). NMR chemical shifts matches the literature values. HR-ESI-MS nor EI-MS was achievable for this compound.

^{1}H NMR (400 MHz, *CDCl$_3$*) δ ppm 5.89 (dd, *J* = 17.2, 10.7 Hz, 1H), 5.36 (d, *J* = 17.2 Hz, 1H), 5.18 (d, *J* = 10.7 Hz, 1H), 3.46 (s, 2H), 2.26 (s, 1H), 1.41 (s, 3H).

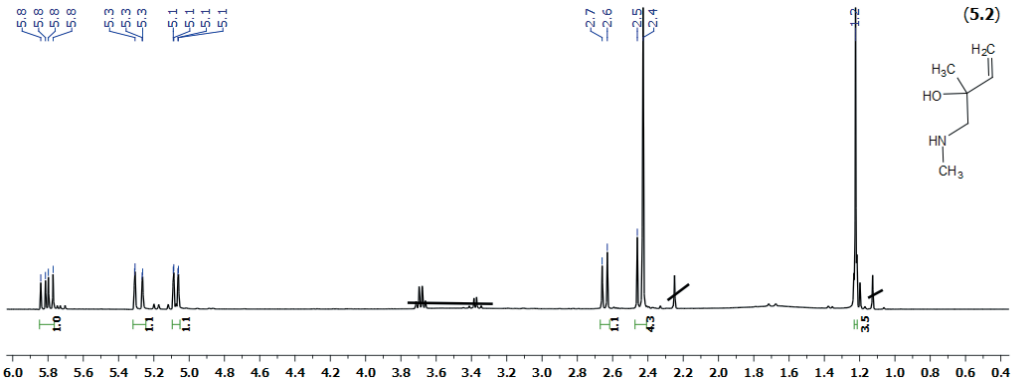
^{13}C NMR (100 MHz, *CDCl$_3$*) δ ppm 141.30, 114.66, 71.78, 44.68, 25.92.



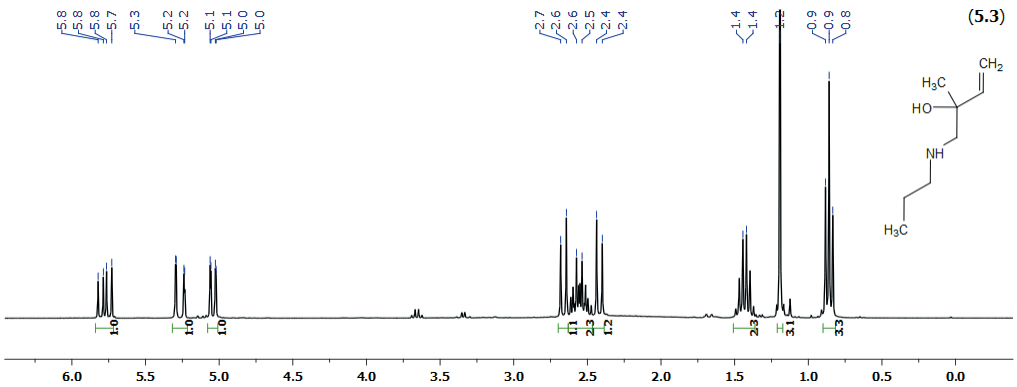
(**5.2**) **2-methyl-1-(methylamino)-3-butene-2-ol:** (NB: This proceeding has previously been described[47]). To a small Schlenk flask aqueous methylamine (20 mL, 40 % wt) was added, the flask was cooled to 0 °C, before (**5.1**) (3 gr., 18.18 mmol) was added dropwise. The Schlenk-flask was sealed and allowed to warm to room temperature, then stirred overnight for 18 hours. To the reaction-mixture was added a saturated sodium hydroxide solution (20 mL, 50 % vol), the organic products were extracted with Et_2O (3 x 40 mL). The combined organic phases were dried with $MgSO_4$, and the solvent removed by rotary evaporation, yielding a clear colorless oil: 1.781 gr. (85%).

^{1}H NMR (400 MHz, *CDCl$_3$*) δ ppm 5.81 (dd, *J* = 17.2, 10.4 Hz, 1H), 5.29 (d, *J* = 17.2 Hz, 1H), 5.08 (d, *J* = 10.4 Hz, 1H), 2.65 (ABX, J_{ab} = 11.6, 1H), 2.44 (ABX, 1H), 2.43 (s, 3H), 1.22 (s, 3H).

^{13}C NMR (100 MHz, *CDCl$_3$*) δ ppm 143.62, 113.00, 71.78, 60.99, 36.76, 25.86. GC-MS (EI): R_t = 6.92 min, m/z = 84 (main fraction). HRMS (ESI+): calcd. for $C_6H_{14}NO^+$ 116.1070, found 116.1066 (MH^+).



(**5.3**) **2-methyl-1-(propylamino)-3-buten-2-ol:** (NB: This proceeding has previously been described[47]). To a flame dried Schlenk flask n-propylamine (20 mL) was added and the flask cooled to 0°C. To this (**5.1**) (4.442 gram, 26.9 mmol) was added dropwise and the flask was heated to n-propylamine's b.p. ~ 50°C before the flask was closed and heated to 60°C. The reaction was stirred at this temperature for 6 hours before it was cooled to room temperature and the solvent reduced to half volume under vacuum. The resulting reaction mixture was partitioned between diethyl ether (40 mL) and aqueous NaOH (15%, 40 mL). The aqueous layer was extracted with diethyl ether (3 x 40 mL) and the combined organic phases were dried with $MgSO_4$, then filtered and the solvent removed on a rotary evaporator. The product was purified by a vial-to-vial vacuum transfer, to yield a colorless liquid: 3.48 gram (90 %).



^{1}H NMR (400 MHz, *CDCl$_3$*) δ ppm 5.78 (dd, *J* = 17.2, 10.8 Hz, 1H), 5.27 (d, *J* = 17.2 Hz, 1H), 5.06 (d, *J* = 10.8 Hz), 2.67 (ABX, J_{ab} = 11.6 Hz, 1H), 2.56 (m, 2H), 2.43 (ABX, J_{ab} = 11.6 Hz, 1H), 1.44 (m, 2H), 1.20 (s, 3H), 0.87 (t, *J* = 7.2 Hz, 3H).

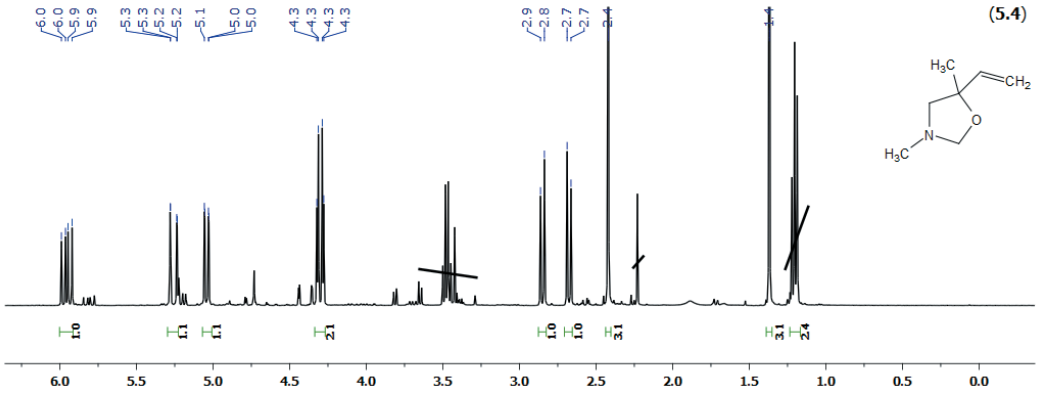
^{13}C NMR (100 MHz, *CDCl$_3$*) δ ppm 143.71, 112.90, 71.49, 58.45, 51.83, 25.78, 23.12, 11.53. GC-MS (EI): R_t = 10.16 min, m/z = 72 (main fraction). HRMS (ESI+): calcd. for $C_8H_{18}NO^+$ 144.1383, found 144.1378 (MH^+).

General procedure for synthesis of oxazolidines

(**5.4 – 5.8**): In a small scintillation vial (20 mL) the starting material (**5.2**) or (**5.3**), (4.5 – 5 mmol) dissolved in Et_2O (5 mL) and cooled to 0°C. The aldehyde* (1.1 eq.) was added; the vial closed and allowed to warm to room temperature. After stirring of the reaction mixture for 30 min, the reaction was dried with $MgSO_4$, filtered and solvents evaporated to yield the corresponding oxazolidine as clear to very pale yellow liquids (these where used without further purification in the cluster experiments). * for product (**5.4**) and (**5.7**) aqueous formaldehyde (37 % wt) was used.

(**5.4**) **3,5-dimethyl-5-vinyloxazolidine:** Yield: 493 mg (88%).
^{1}H NMR (400 MHz, *CDCl$_3$*) δ ppm 5.95 (dd, *J* = 17.2, 10.4 Hz, 1H), 5.25 (d, *J* = 17.2 Hz, 1H), 5.03 (d, *J* = 10.4 Hz, 1H), 4.31 (ABX, J_{ab} = 4.0 Hz, 1H), 4.27 (ABX, J_{ab} = 4.0 Hz, 1H), 2.84 (ABX, J_{ab} = 10.0 Hz, 1H), 2.67 (ABX, J_{ab} = 10.0 Hz, 1H), 2.41 (s, 3H), 1.36 (s, 3H).

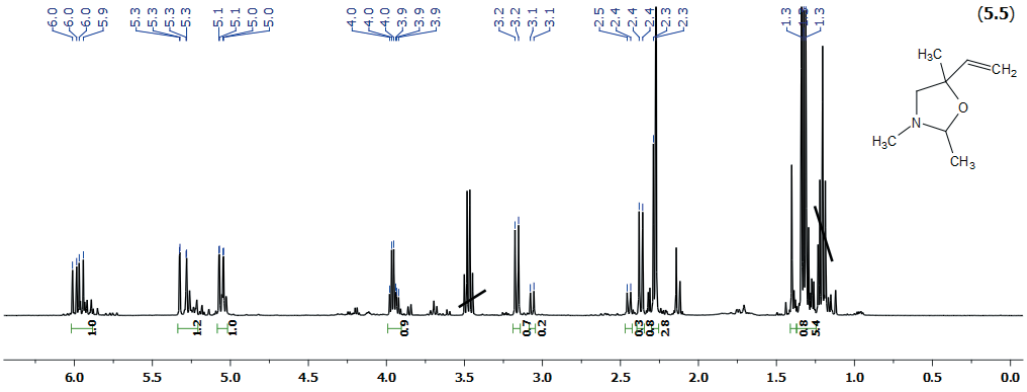
^{13}C NMR (100 Mz, *CDCl$_3$*) δ ppm 142.97, 112.02, 87.76, 81.02, 65.54, 40.69, 25.66. GC-MS (EI): R_t = 7.37 min, m/z = 127 (M^+), 112 (main fraction), 96, 84, 72, 56, 42. HRMS (ESI+): calcd. for $C_7H_{14}NO^+$ 128.1070, found 128.1065 (MH^+).



(**5.5**) **2,3,5-trimethyl-5-vinyloxazolidine:** Yield: 540 mg (87%) as a (1:3) mixture of diastereomers.

^{1}H NMR (400 MHz, *CDCl$_3$*) Major diastereomer: δ ppm 5.97 (dd, *J* = 17.2, 10.4 Hz, 1H), 5.30 (dd, *J* = 17.2, 1.2 Hz, 1H), 5.05 (dd, *J* = 10.4, 1.2 Hz, 1H), 3.95 (q, *J* = 5.2 Hz, 1H), 3.16 (ABX, J_{ab} = 9.2 Hz, 1H), 2.36 (ABX, J_{ab} = 9.2 Hz, 1H), 2.26 (s, 3H), 1.33 (s, 3H), 1.31 (d, J = 5.2 Hz, 3H). Minor diastereomer: δ ppm 5.92 (dd, *J* = 17.2, 10.8 Hz, 1H), 5.23 (d, *J* = 17.2 Hz, 1H), 5.03 (d, *J* = 10.8 Hz, 1H), 3.92 (q, *J* = 5.2 Hz, 1H), 3.06 (ABX, J_{ab} = 9.2, 1H), 2.44 (ABX, J_{ab} = 9.2 Hz, 1H), 2.28 (s, 3H), 1.39 (s, 3H), 1.29 (d, *J* = 5.6 Hz, 3H).

^{13}C NMR (100 MHz, *CDCl$_3$*) δ ppm 143.51, 112.23, 93.25, 79.45, 67.10, 37.90, 25.31, 18.86. GC-MS (EI): Rt_{major} = 7.74 min, Rt_{minor} = 7.39 min, m/z = 140 (M^+), 98, 71 (main fraction), 56, 42, 27. HRMS (ESI+): calcd. for $C_8H_{16}NO^+$ 142.1226, found 142.1221 (MH^+).

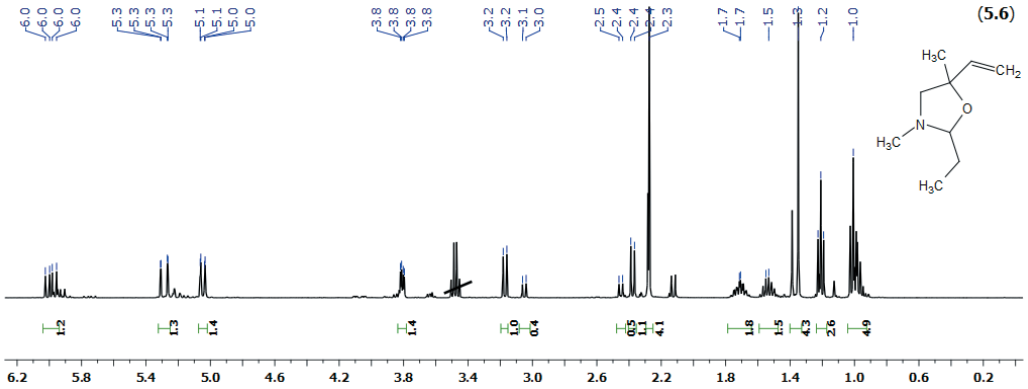


(**5.6**) **2-ethyl-3,5-dimethyl-5-vinyloxazolidine:** Yield: 616 mg (90%) as a (1:3) mixture of diastereomers.

^{1}H NMR (400 MHz, *CDCl$_3$*) Major diastereomer: δ ppm 5.98 (dd, *J* = 17.2, 10.4 Hz, 1H), 5.28 (dd, *J* = 17.2, 1.2 Hz, 1H), 5.04 (dd, *J* = 10.4, 1.2 Hz, 1H), 3.80 (m, 1H), 3.16 (ABX, J_{ab} = 9.2 Hz, 1H), 2.37 (ABX, J_{ab} = 9.2 Hz, 1H), 2.27 (s, 3H), 1.76 – 1.65 (m, 1H), 1.58 – 1.47 (m, 1H), 1.34 (s, 3H), 1.00 (t, *J* = 7.6 Hz, 3H). Minor diastereomer: δ ppm 5.93 (dd, *J* = 17.2, 10.8 Hz, 1H), 5.24 (d, *J* = 17.2, 1H), 5.04 (d, *J* = 10.8, 1H), 3.80 (m, 1H), 3.04 (ABX, J_{ab} = 8.8 Hz, 1H), 2.44 (ABX, J_{ab} = 8.8 Hz, 1H), 2.28 (s, 3H), 1.76 – 1.65 (m, 1H), 1.58 – 1.47 (m, 1H), 1.38 (s, 3H), 0.97 (t, *J* = 6.8 Hz, 3H).

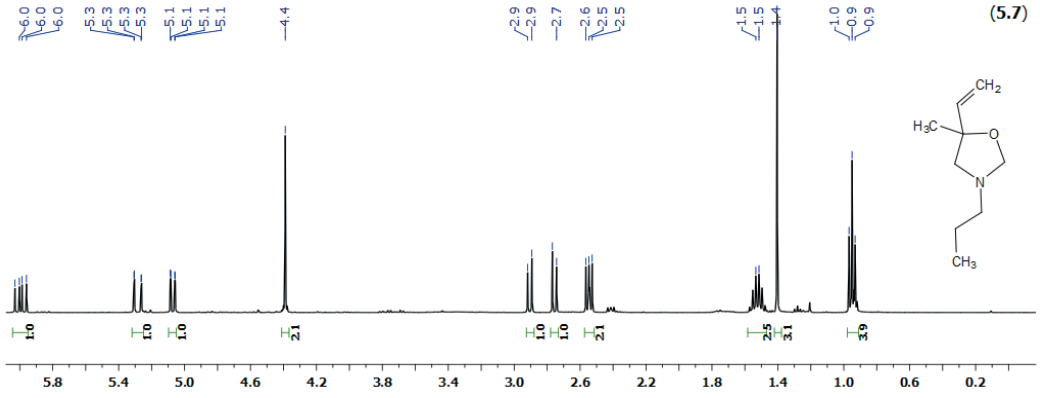
^{13}C NMR (100 MHz, *CDCl$_3$*) δ ppm 143.70, 112.10, 98.14, 79.25, 67.11, 38.37, 25.82, 25.30, 8.92. GC-MS (EI): R_t (major) = 9.37 min, R_t (minor) = 9.05 min, m/z =

154 (M^+), 140, 126 (major fraction), 98, 85, 70, 56, 42, 27. HRMS (ESI+): calcd. for $C_9H_{18}NO^+$ 156.1383, found 156.1379 (MH^+).



(**5.7**) **3-propyl-5-methyl-5-vinyloxazolidine:** Yield: 709 mg (91%).
^{1}H NMR (400 MHz, *CDCl$_3$*) δ ppm 5.95 (dd, *J* = 17.2, 10.8 Hz, 1H), 5.24 (dd, *J* = 17.2, 1.2 Hz, 1H), 5.03 (dd, *J* = 10.8, 1.2 Hz, 1H), 4.35 (s, 2H), 2.86 (ABX, J_{ab} = 10.4 Hz, 1H), 2.71 (ABX, J_{ab} = 10.4 Hz, 1H), 2.50 (t, *J* = 8.0 Hz, 2H), 1.48 (m, 2H), 1.36 (s, 3H), 0.91 (t, *J* = 7.6 Hz, 3H).

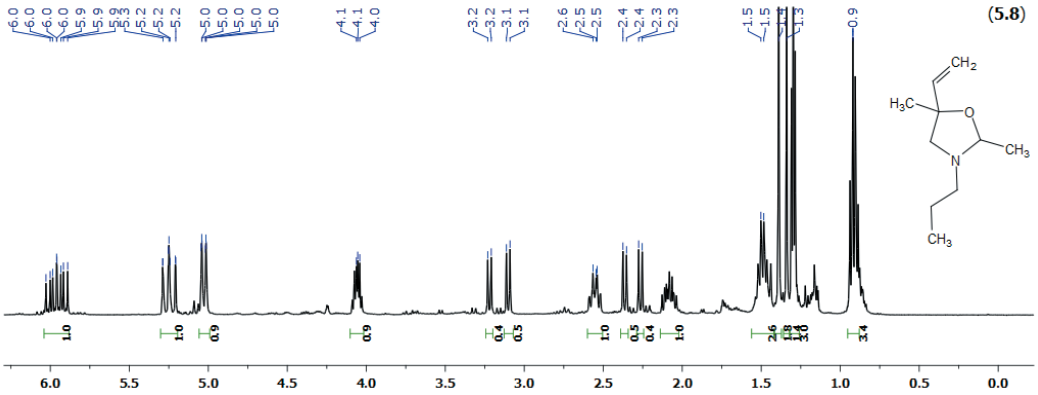
^{13}C NMR (100 MHz, *CDCl$_3$*) δ ppm 142.99, 112.03, 86.22, 80.50, 63.81, 56.31, 25.58, 18.42, 11.76. GC-MS (EI): R_t = 10.52 min, m/z = 154 (M^+), 140, 126, 84 (main fraction), 69, 57, 43, 27. HRMS (ESI+): calcd. for $C_9H_{18}NO^+$ 156.1383, found 156.1377 (MH^+).



(**5.8**) **3-propyl-2,5-dimethyl-5-vinyloxazolidine:** Yield: 673 mg (87%) as a (1:1) mixture of diastereomers.

^{1}H NMR (400 MHz, *CDCl$_3$*) δ ppm both diastereomers: 5.99 (dd, J = 17.2, 10.8 Hz, 1H), 5.92 (dd, *J* = 17.2, 10.8 Hz, 1H), 5.25 (m, 2H), 5.03 (m, 2H), 4.06 (m, 2H), 3.22 (ABX$_1$, J_{ab} = 9.2 Hz, 1H), 3.10 (ABX$_2$, J_{ab} = 8.8 Hz, 1H), 2.55 (m, 2H), 2.36 (ABX$_1$, J_{ab} = 9.2 Hz, 1H), 2.26 (ABX$_2$, J_{ab} = 8.8 Hz, 1H), 2.13 – 2.04 (m, 2H), 1.52 – 1.44 (m, 4H), 1.39 (s, 3H), 1.34 (s, 3H), 1.31 – 1.29 (m, 6H), 0.94 – 0.89 (m, 6H).

GC-MS (EI): R$_t$ = 10.45 – 10.55 min, m/z = 166 (M$^+$), 154, 140, 126, 99, 84 (main fraction), 69, 56, 41, 27. HRMS (ESI+): calcd. for $C_{10}H_{20}NO^+$ 170.1539, found 170.1536 (MH$^+$).



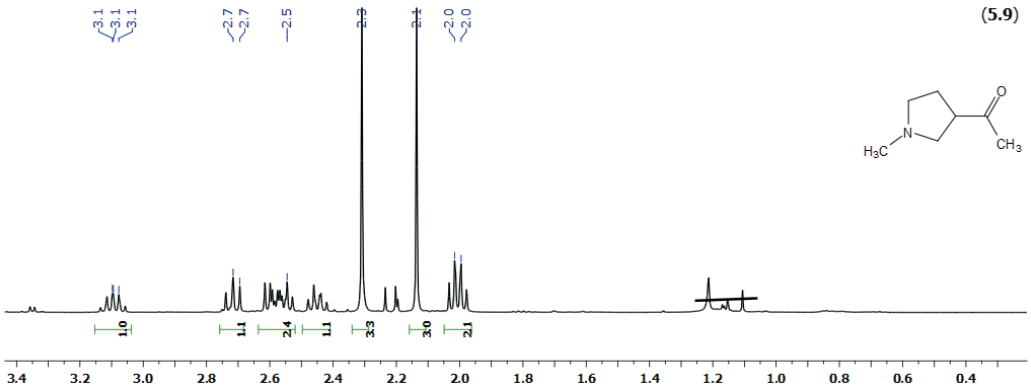
Acid mediated aza-Cope Mannich experiments

General procedure for the synthesis of 3-acetyl-pyrrolidines. In small pop-cap vials (+)-camphor sulphonic acid (100 mg, 0.43 mmol) was dissolved in dry benzene (2 mL). To this solution the oxazolidine (**5.4** – **5.8**) (1 eq., 0.43 mmol) was added. The vials were closed, tightened and then heated to 60^{o}C and left stirring for 24 hours. The reaction mixtures were then added aqueous sodium hydroxide (6M, 2 mL) and then extracted with Et_2O (3 x 2 mL). The combined organic phases were dried with $MgSO_4$, filtered and evaporated on a rotary evaporator to yield the 2-acetylpyrrolidines as clear liquids. The conversion was determined by GC-MS.

(**5.9**) **N-methyl-3-acetylpyrrolidine:** Conversion: 100%. Yield: 48 mg (88%).

^{1}H NMR (400 MHz, *CDCl$_3$*) δ ppm 3.14 – 3.06 (m, 1H), 2.74 – 2.70 (m, 1H), 2.62 – 2.53 (m, 2H), 2.48 – 2.42 (m, 1H), 2.31 (s, 3H), 2.14 (s, 3H), 2.03 – 1.98 (m, 2H).

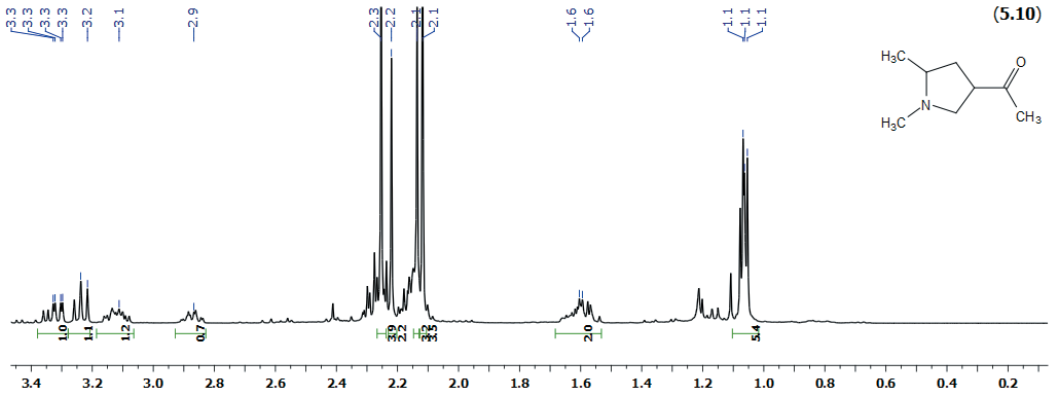
^{13}C NMR (100 MHz, *CDCl$_3$*) δ ppm 208.71, 57.62, 55.98, 50.77, 41.84, 28.53, 27.03. GC-MS (EI): R$_t$ = 10.26 min, m/z = 127 (M$^+$), 112, 84, 67, 57, 42 (main fraction), 27.



(**5.10**) **N-methyl-3-acetyl-5-methylpyrrolidine:** Conversion: 100%. Yield: 47 mg (87%) as a (3:5) mixture of diastereomers measured by GC-MS.

^{1}H NMR (400 MHz, *$CDCl_3$*) both diastereomers δ ppm 3.36 – 3.30 (m, 1H), 3.26 – 3.22 (m, 1H), 3.16 – 3.08 (m, 1H), 2.91 – 2.84 (m, 1H), 2.30 – 2.22 (m, 3H), 2.25 ($s_{majordiastereomer}$, 3H), 2.22 ($s_{minordiastereomer}$, 3H), 2.18 – 2.10 (m, 3H), 2.14 ($s_{minordiastereomer}$, 3H), 2.12 ($s_{majordiastereomer}$, 3H), 1.66 – 1.54 (m, 2H), 1.08 – 1.05 (m, 6H).

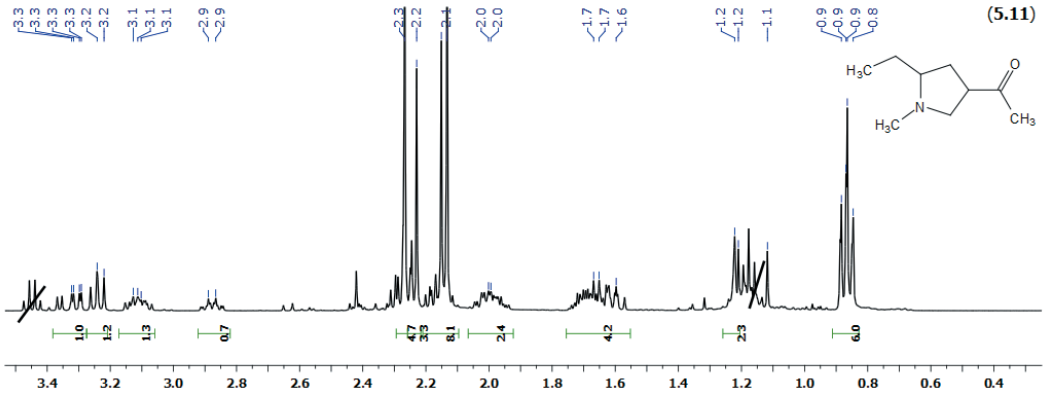
^{13}C NMR (100 MHz, *$CDCl_3$*) both diastereomers δ ppm 208.83 (1C), 208.77 (1C), 61.65 (1C), 60.89 (1C), 58.85 (1C), 57.79 (1C), 48.37 (1C), 48.16 (1C), 39.68 (2C), 36.09 (1C), 35.28 (1C), 29.07 (1C), 27.64 (1C), 18.40 (1C), 18.01 (1C). GC-MS (EI): R_t (major) = 10.95 min, R_t (minor) = 11.10 min, m/z (both) = 141 (M^+), 126, 108, 98, 82 (main fraction), 71, 56, 44, 42, 28, 27.



(5.11) N-methyl-3-acetyl-5-ethylpyrrolidine: Conversion: 95%. Yield: 45 mg (90%) as a (1:2) mixture of diastereomers measured by GC-MS.

^{1}H NMR (400 MHz, *CDCl$_3$*) both diastereomers δ ppm 3.37 (m, 1H), 3.26 – 3.22 (m, 1H), 3.15 – 3.07 (m, 1H), 2.91 – 2.84 (m, 1H), 2.32 – 2.25 (m, 2H), 2.27 ($s_{majordiastereomer}$, 3H), 2.23 ($s_{minordiastereomer}$, 3H), 2.20 – 2.15 (m, 2H), 2.15 ($s_{minordiastereomer}$, 3H), 2.13 ($s_{majordiastereomer}$, 3H), 2.05 – 1.94 (m, 2H), 1.74 – 1.57 (m, 4H), 1.22 – 1.12 (m, 2H), 0.89 – 0.85 (m, 6H).

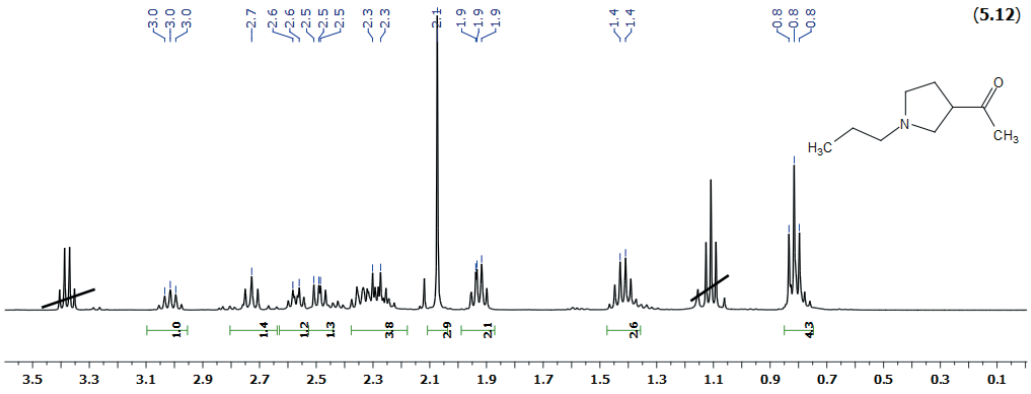
^{13}C NMR (100 MHz, *CDCl$_3$*) both diastereomers δ ppm 208.97 (1C), 208.83 (1C), 67.96 (1C), 67.15 (1C), 59.08 (1C), 58.09 (1C), 48.57 (1C), 48.15 (1C), 39.97 (2C), 33.29 (1C), 32.67 (1C), 29.64 (1C), 29.13 (1C), 26.10 (1C), 25.57 (1C), 10.43 (2C). GC-MS (EI): R_t (major) = 12.63 min, R_t (minor) = 12.75 min, m/z (both) = 155 (M^+), 108, 82 (main fraction), 67, 55, 42, 27.



(**5.12**) **N-propyl-3-acetylpyrrolidine:** Conversion: 100%. Yield: 55 mg (91%).

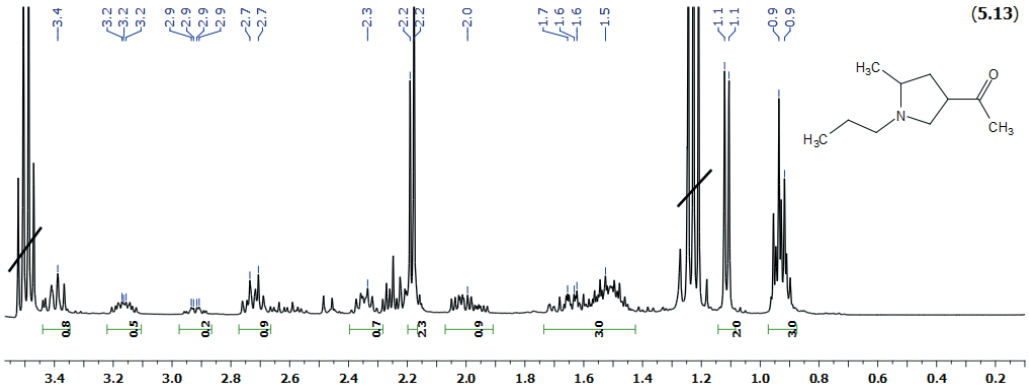
^{1}H NMR (400 MHz, *CDCl$_3$*) δ ppm 3.12 – 3.04 (m, 1H), 2.82 – 2.77 (m, 1H), 2.66 – 2.61 (m, 1H), 2.57 – 2.53 (m, 1H), 2.44 – 2.29 (m, 3H), 1.14 (s, 3H), 2.02 – 1.96 (m, 2H), 1.53 – 1.44 (hex, *J* = 7.6 Hz, 2H), 0.88 (t, *J* = 7.6 Hz, 3H).

^{13}C NMR (100 MHz, *CDCl$_3$*) δ ppm 208.88, 58.04, 55.57, 53.91, 50.23, 28.51, 26.41, 21.97, 11.96. GC-MS (EI): R_t = 13.12 min, m/z = 155 (M^+), 126 (main fraction), 112, 84, 70, 55, 43, 27.



(**5.13**) **N-propyl-3-acetyl-5-methylpyrrolidine:** Conversion: 90%. Yield: 87%.

The mixture of compounds made it impossible to assign NMR peaks.
1H NMR (400 MHz, $CDCl_3$):

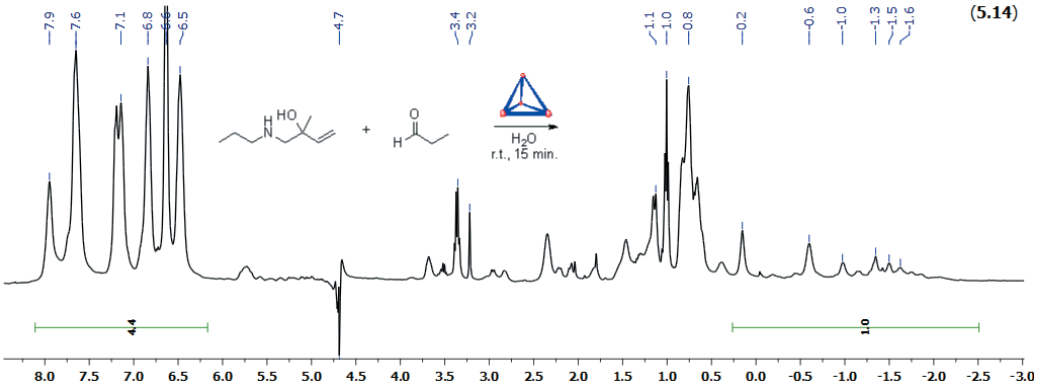


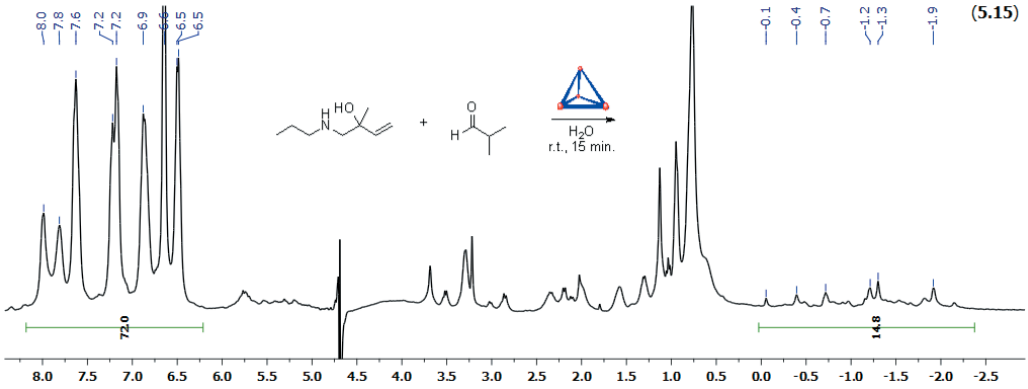
GC-MS (EI): R_t = 13.59 min, m/z = 169 (M^+), 154, 140 (main fraction), 126, 110, 96, 82, 68, 56, 43.

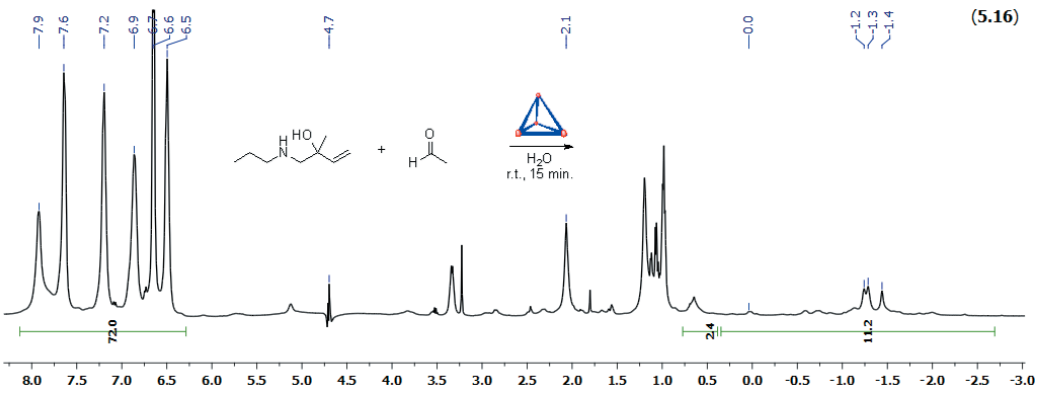
Assembly mediated aza-Cope Mannich experiments.

(**5.14** – **5.16**) **2-methyl-1-(propylamino)-3-buten-2-ol** + **aldehydes:** General procedure for these cluster experiments: In the glovebox (**1**) (20 mg) was weighed out in small capped vial. In a second vial (**5.3**) (6 uL) and the aldehyde (12 uL) where syringed into a degassed (by N_2 bubbling) solution of H_2O (1000 uL) and D_2O (100 uL). A noteworthy precipitate was formed, but after 5-10 min of gently agitation of the vial the reaction mixture became clear. Half of this substrate solution was then transferred to the vial with the cluster (**1**) - the mixture was shaken until dissolution of the cluster occurred. The samples (the cluster + substrates and substrate samples) were then transferred to a medium walled NMR tube and analyzed by NMR spectroscopy. The upfield areas of the spectra are shown on page 14 and 15.

^{1}H NMR (400 mHz, D_2O) Watergate Solvent Supression.



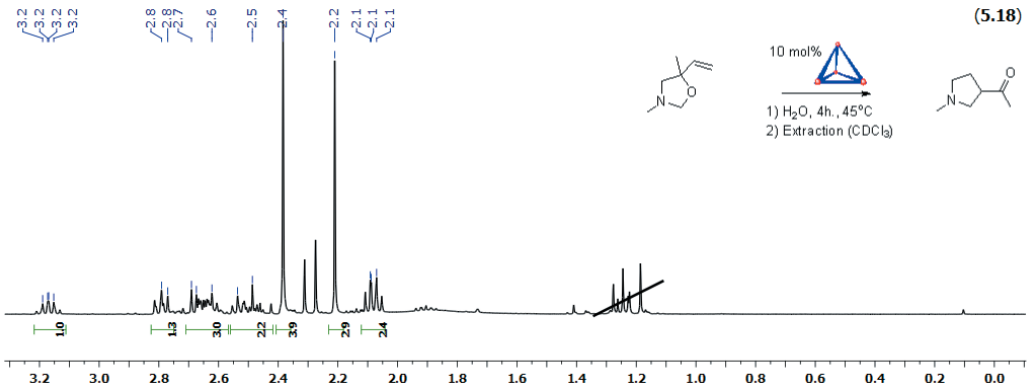




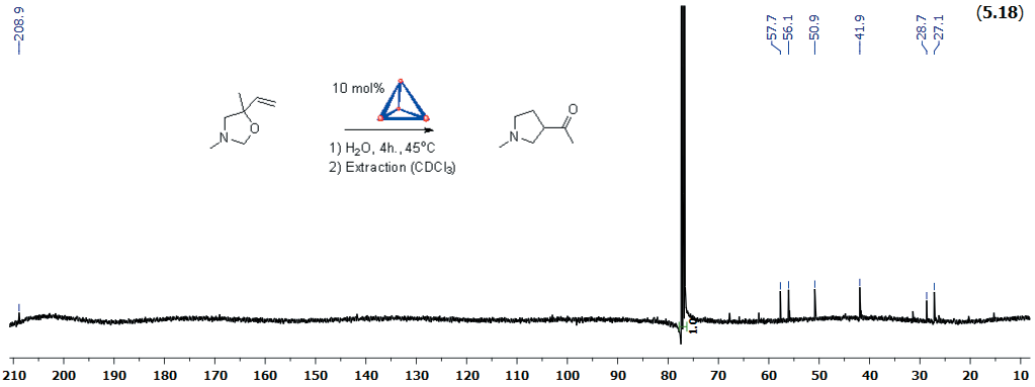
(**5.18 – 5.19**) **N-alkyl-2-acetylpyrrolidine (Upscale x 5):** In a small pop-cap vial (**5.4**) (37 mg, 289 umol) or (**5.7**) (44 mg, 283 umol) was dissolved in H_2O (2.5 mL, 100 mM K_2CO_3, pH = 11.5) with gentle agitation of the mixture. Total dissolution took between 5-10 minutes, after which the solution was degassed by N_2 bubbling. To this mixture was then added 10 mol% (**1**) (104 mg or 102 mg), the vial closed and heated to 45 ^{o}C for 4 hours. After reaction was complete, Et_4NBr was added to the reaction mixture (12.5 mg, 59 umol, 2 eq.) and the solution extracted with Et_2O (3 x 3 mL). The combined organic layers were dried with $MgSO_4$, filtered and the solvent evaporated on a rotary evaporator.

^{1}H NMR spectroscopy experiments showed a mixture of starting material and pyrrolidine product. The spectra are shown beneath.

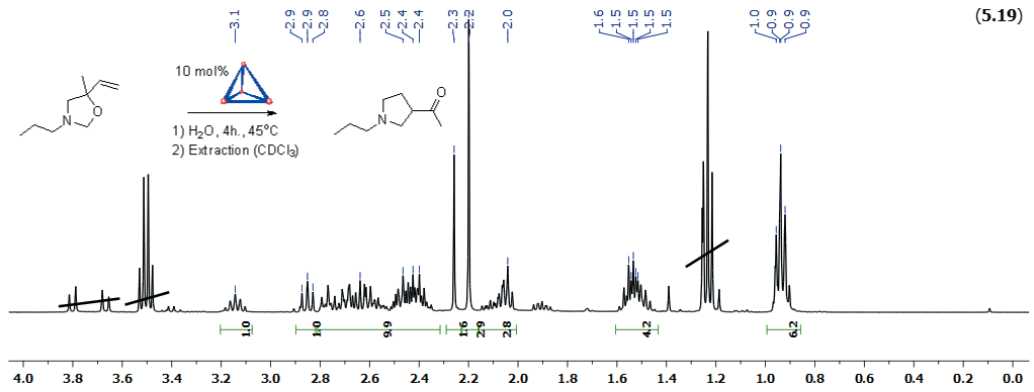
^{1}H-NMR (400 MHz, $CDCl_3$).



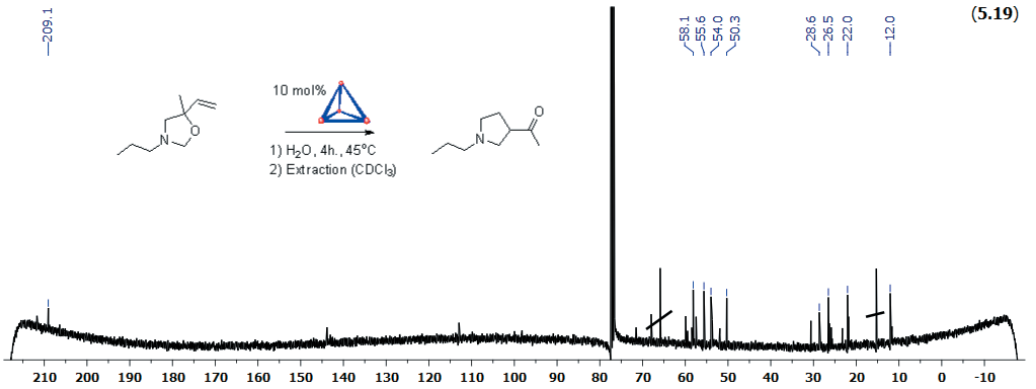
^{13}C-NMR (400 MHz, $CDCl_3$).



^{1}H-NMR (**5.19**).



^{13}C-NMR (**5.19**).



(**5.20 – 5.24**) **General procedure for catalytic reactions with oxazolidines.** In the glovebox (**1**) (20 mg, approx. 5.6 umol) was weighed-out in small capped vial. In other pop-cap vials the oxazolidines (**5.4**, 14.2 mg, 112 umol); (**5.5**, 15.8 mg, 112 umol); (**5.6**, 17.2 mg, 112 umol); (**5.7**, 19.0 mg, 122 umol); (**5.8**, 17.8 mg, 106 umol) were dissolved in buffered H_2O (1000 uL, 100 mM K_2CO_3, pH = 11.5) and D_2O (100 uL) with gentle agitation. The reagent solution was degassed by bubbling N_2 gas through the solution for 10 minutes, subsequently half of the solution (550 uL) was transferred to the vial with (**1**) and mixed until homogenous.

The remaining reagent solution and the reaction mixture were transferred to separate medium-walled NMR tubes and analyzed. The samples were heated to 45^{o}C in a water bath and the reactions followed by NMR spectroscopy. After the reaction was complete (for the exp. **5.20** and **5.23**), Et_4N^+ (11.2 umol) was added to the cluster experiments, which were then treated with Et_2O (3 x 3 mL). The combined organic phases were dried with $MgSO_4$, filtered and evaporated. The background samples were treated the same way. Subsequently the products were analyzed by GC-MS to detect the conversion and result of the catalytic reactions.

Identification of pyrrolidine product was based on GC-retention time and EI molecular fraction pattern; results are listed in table below.

Exp.	*Reaction conditions*	*Rt. and diastereomeric ratio*	*Conversion.*
(5.20)	3 h., 45^{o}C	Rt. = 10.27 min.	> 95 %
(5.21)	9h, 45^{o}C + 9h., 60^{o}C	Rt. = 10.95 and 11.11 min. (1:1)	65 %
(5.22)	9h, 45^{o}C + 9h., 60^{o}C	Rt. = 12.63 and 12.75 min. (1:1)	79 %
(5.23)	3 h., 45^{o}C	Rt. = 13.15 min.	> 95 %
(5.24)	31 h., 45^{o}C	Rt. = 13.62 min. (1:1)	50 %

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